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Développement de la iontophorèse comme axe thérapeutique des atteintes microcirculatoires dans la sclérodermie systémique

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THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ DE GRENOBLE

Spécialité : Ingénierie de la santé, la cognition et l'environnement/ Biotechnologie, Instrumentation, Signal

Présentée par

Sophie BLAISE

Thèse dirigée par Professeur Jean Luc CRACOWSKI

Préparée au sein du Centre d'Investigation Clinique du Centre Hospitalier et Universitaire de Grenoble

Dans l'École Doctorale INGÉNIERIE POUR LA SANTÉ, LA COGNITION ET L'ENVIRONNEMENT

**Développement de la iontophorèse
comme axe thérapeutique des
atteintes microcirculatoires dans la
sclérodermie systémique.**

Thèse soutenue publiquement le **10 novembre 2011**

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RESUME

La sclérodémie systémique (ScS) est une maladie qui peut engager le pronostic vital des malades de façon très rapide. La pathogénie de la maladie reste encore obscure mais est liée aux atteintes vasculaires probablement en lien avec la fibrose cutanée. La thérapeutique de cette maladie est difficile. Aucun traitement étiologique n'existe et la prise en charge est plutôt axée sur les traitements des différentes atteintes. La iontophorèse cutanée est un dispositif qui permet la diffusion de molécules en solution à travers la peau grâce à une stimulation électrique de manière non invasive. Cette technique a été utilisée initialement comme test physiologique. Notre objectif est d'évaluer et de développer la iontophorèse thérapeutique, notamment avec des molécules vasodilatatrices, pour pouvoir l'utiliser dans des applications thérapeutiques telles que la prise en charge des ulcérations digitales (ou troubles trophiques cutanés) de la ScS.

Trois parties ont été développées. La première partie consiste en l'association de deux molécules vasodilatatrices (une per os, le sildenafil, et en iontophorèse, le nitroprussiate de sodium (SNP), en étudiant la tolérance de l'association et son effet sur le flux vasculaire cutané chez le volontaire sain (étude INFLUX-VS). La deuxième partie correspond à un screening de molécules ayant une action vasodilatatrice et étant délivrées en iontophorèse chez le rat : l'étude INFLUX-RAT conclue à l'obtention d'une vasodilatation cutanée chez le rat avec les analogues de la prostacycline. La troisième partie correspond à l'évaluation chez le volontaire sain de la iontophorèse des analogues de la prostacycline : l'étude INFLUX-IT-VS conclue à une dilatation du flux sanguin cutané avec le tréprostinil et avec une bonne tolérance cutanée et systémique.

La dernière partie correspond aux avancées parallèles des travaux tant dans le domaine de la reproductibilité des techniques d'acquisition des signaux du flux sanguin cutané que les études réalisées avec les patients sclérodermiques à qui on pourrait espérer un jour voir proposer une iontophorèse thérapeutique.

Development of the iontophoresis as a therapeutic area of the microvascular in the systemic scleroderma

ABSTRACT

Systemic scleroderma (ScS) is a rare disease that may be associated with a poor prognosis. The pathogenesis of the disease remains still unclear but comprises vascular abnormalities related to skin fibrosis. ScS disease treatment is difficult. No etiologic therapy is available and patient's management is rather centred on the treatments of the different organs failure symptoms. Skin iontophoresis is a non invasive technique, which allows a transcutaneous diffusion of molecules in solution thanks to an electrical stimulation. This technique was initially used as physiological test. Our objective is to test and to develop therapeutic iontophoresis, in particular with molecules possessing vasodilator properties. The final objective is to use it in therapeutic applications such as the treatment of ScS digital ulcerations. Three parts will be developed in the present work. The first part describes the association of two vasodilator drugs (sildenafil used per os, associated with sodium nitroprusside used through cathodal iontophoresis). We studied the safety of the association and its effect on the skin vascular flow in healthy volunteers (INFLUX-VS study). The second part describes the screening of vasodilator drugs delivered through cutaneous iontophoresis in rats (INFLUX-RAT study). The aim of the study was to select the more potent drugs in term of maximal rat skin vasodilatation. The more potent drugs were prostacyclins analogues. The third part describes the iontophoresis of prostacyclins analogues in healthy volunteers:

the INFLUX-IT-VS study. The more potent cutaneous vasodilatation was observed with treprostinil, with a good skin and systemic tolerance. The last part describes the reproducibility of the techniques used to quantify skin blood flow along with studies using these techniques in scleroderma patients. These are required to enable a reproducible evaluation of the effect of skin iontophoresis in patients with scleroderma

Mots clés: iontophorèse, microcirculation, sclérodémie systémique, analogues des prostacyclines

Key-words: iontophoresis, microcirculation, systemic scleroderma, prostacyclin analogues.

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INTRODUCTION

1.1 L'atteinte microcirculatoire dans la sclérodémie systémique

La sclérodémie systémique se caractérise essentiellement par deux types de lésions la vasculopathie et la fibrose. Les lésions vasculaires touchent la microcirculation et la macrocirculation (artères viscérales ou des artères des membres). L'atteinte vasculaire est une atteinte précoce dans la ScS et précède la fibrose mais les deux seraient liées (*figure 1*). La microangiopathie sclérodermique se traduit par des anomalies morphologiques et fonctionnelles. Elle est responsable de différentes atteintes viscérales: le phénomène de Raynaud, les troubles trophiques des extrémités (ulcères digitaux et ischémie distale) mais aussi la néphroangiopathie, l'hypertension artérielle pulmonaire ou les atteintes cardiaques. La fibrose cutanée et viscérale est elle liée à une accumulation de collagène via une stimulation des fibroblastes, avec une accumulation notamment de collagène dans le derme profond.

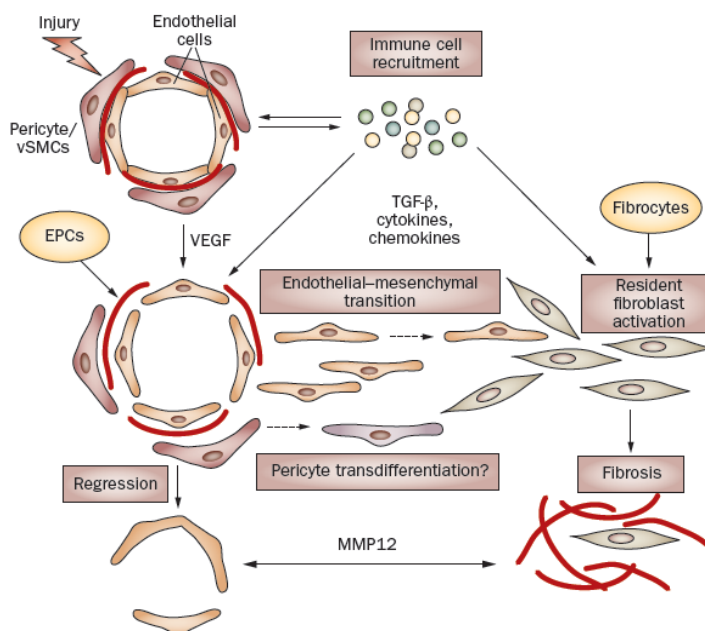


Figure 1 : possibles liens entre la vasculopathie et la fibrose dans la ScS.

EPCs : endothelial progenitor cells ; MMP12 : matrix metalloproteinase 12 ; TGF β : Tumor Growth Factor β ; VEGF : Vascular Endothelial Growth Factor ; vSMCs : vascular smooth muscular cells (Trojanowska)

1.1.1 Physiopathologie de l'atteinte microcirculatoire.

Plusieurs hypothèses physiopathologiques sont incriminées et elles sont sans doute multiples. La production accrue de protéines de la matrice extracellulaire par les fibroblastes résulte d'interactions anormales entre les cellules endothéliales, les cellules mononuclées (lymphocytes et monocytes) et les fibroblastes, dans un contexte d'hyperréactivité vasculaire et d'hypoxie tissulaire. Les cellules mononuclées seraient à l'origine des autres dysfonctionnements cellulaires par le biais de cytokines. Le mécanisme initiateur serait cependant vasculaire, comme en témoigne la précession de la maladie par un syndrome de Raynaud.

Les lésions vasculaires, comme la stimulation fibroblastique, seraient secondaires à une dysimmunité. Cette dernière implique à la fois les cellules T, B ainsi que les cytokines et facteurs de croissance. Les lésions vasculaires seraient liées à une hypersécrétion de certaines

cytokines, elle-même en rapport avec une activation des lymphocytes des macrophages. L'activation et la dégranulation mastocytaire souvent observées dans les tissus biopsiés participent vraisemblablement à l'activation de la cellule endothéliale.

La fibrose serait induite par une activation fibroblastique due à une hypersécrétion de cytokines. L'activation fibroblastique aboutit à l'accumulation de collagène, source de fibrose tissulaire. C'est essentiellement le TGF β (Tumor Growth Factor β) synthétisé par les cellules monocytaires qui favorise la prolifération fibroblastique et la synthèse du collagène. Il stimule aussi la production de PDGF (Platelet Derived Growth Factor). Ce PDF est vasoconstricteur et chimiotactique pour les fibroblastes. Le TNF α (Tumor Necrosis Factor α) semble aussi intervenir par son effet mitogène pour les fibroblastes et être cytotoxique pour la cellule endothéliale. Un autre mécanisme évoqué pour expliquer l'excès de collagène serait une perte du feedback négatif physiologique exercé par un résidu peptidique lors de la synthèse collagène.

La participation immunologique est évidente comme en témoigne l'infiltration lymphocytaire T constituée de cellules T activées sécrétant différentes cytokines toxiques pour l'endothélium vasculaire. L'activation lymphocytaire B explique la fréquence des auto-anticorps associés.

Les taux élevés de VEGF (Vascular Endothelial Growth Factor) et d'autres médiateurs proangiogéniques dérivant des cellules immunes activées faciliteraient la prolifération des cellules endothéliales et des péricytes dans l'attente d'une restauration des vaisseaux altérés. Pour des raisons mal comprises mais qui impliqueraient une altération de l'équilibre entre les médiateurs pro angiogéniques et anti angiogéniques, les propriétés intrinsèques des cellules endothéliales et la sécrétion par les fibroblastes de facteurs anti angiogéniques (par exemple MMP12 (matrice métalloprotéinase 12)), le processus de réparation vasculaire n'aboutit pas et conduit à une régression des vaisseaux (*figure 2*).

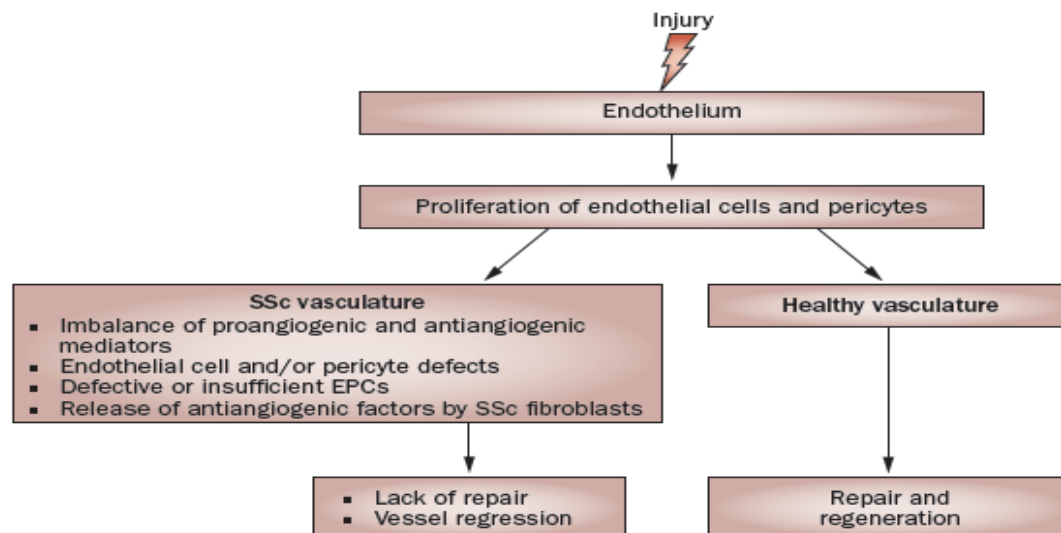


Figure 2 : Représentation schématique des mécanismes potentiellement impliqués dans la néoangiogénèse dans la ScS. Chez le sujet sain, les dommages endothéliaux conduisent à une régénération vasculaire. Dans la ScS, la réponse à la phase précoce de la maladie est une prolifération des cellules endothéliales et des péricytes, mais le processus de réparation est altéré conduisant à une régression des vaisseaux. EPCs= cellules progénitrices endothéliales. (Trojanowska)

En présence de TGF- β ou d'autres médiateurs immuns (telles que les cytokines et les chemokines), ou les deux, les cellules endothéliales pourraient acquérir un phénotype de migration vers une transition de cellules endothéliales à des cellules mésenchymateuses et pénétreraient dans les tissus environnants où elles entreraient ensuite dans une différenciation pour une production de collagène. De même, les péricytes peuvent se différencier en fibroblastes ou myofibroblastes et produire du collagène. Dans la ScS, les propriétés anormales intrinsèques des cellules endothéliales et des péricytes pourraient entraîner ce type de remaniements. Les fibroblastes activés ou les fibrocytes qui entrent dans les tissus atteints sont également une source additionnelle de production de collagène qui contribue à la production de fibrose (*Trojanowska*).

L'expression aberrante de facteurs de transcription endothéliaux, incluant Fra2, Fli1, le « peroxisome proliferator-activated receptor γ » et GATA-6 qui sont associés à la ScS pourraient contribuer aux défauts vasculaires. (*Trojanowska*)

1.1.2 Anomalies structurelles de la vasculopathie

L'atteinte vasculaire de la sclérodémie systémique s'accompagne de lésions artériolaires et capillaires. La participation endothéliale n'est plus à démontrer. L'activation des cellules endothéliales est précoce et aboutit à la micro angiopathie sclérosante. Le remodelage vasculaire est un aspect morphologique important. Le modèle d'angiogénèse schématiquement décrit est altéré dans la ScS (*figure 3*).

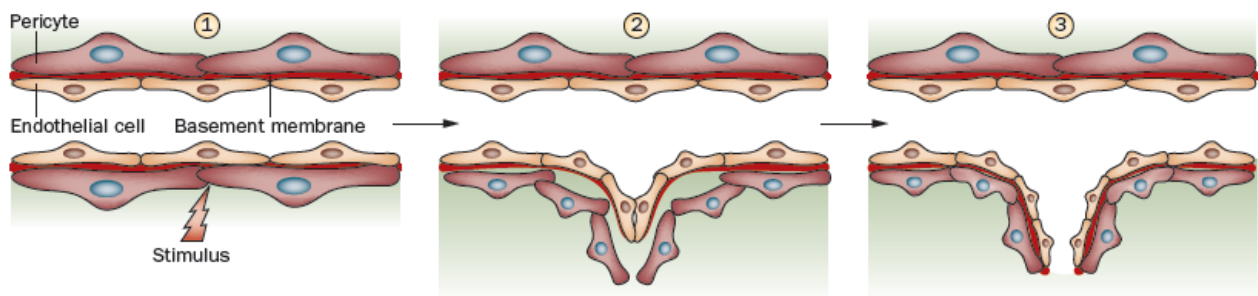
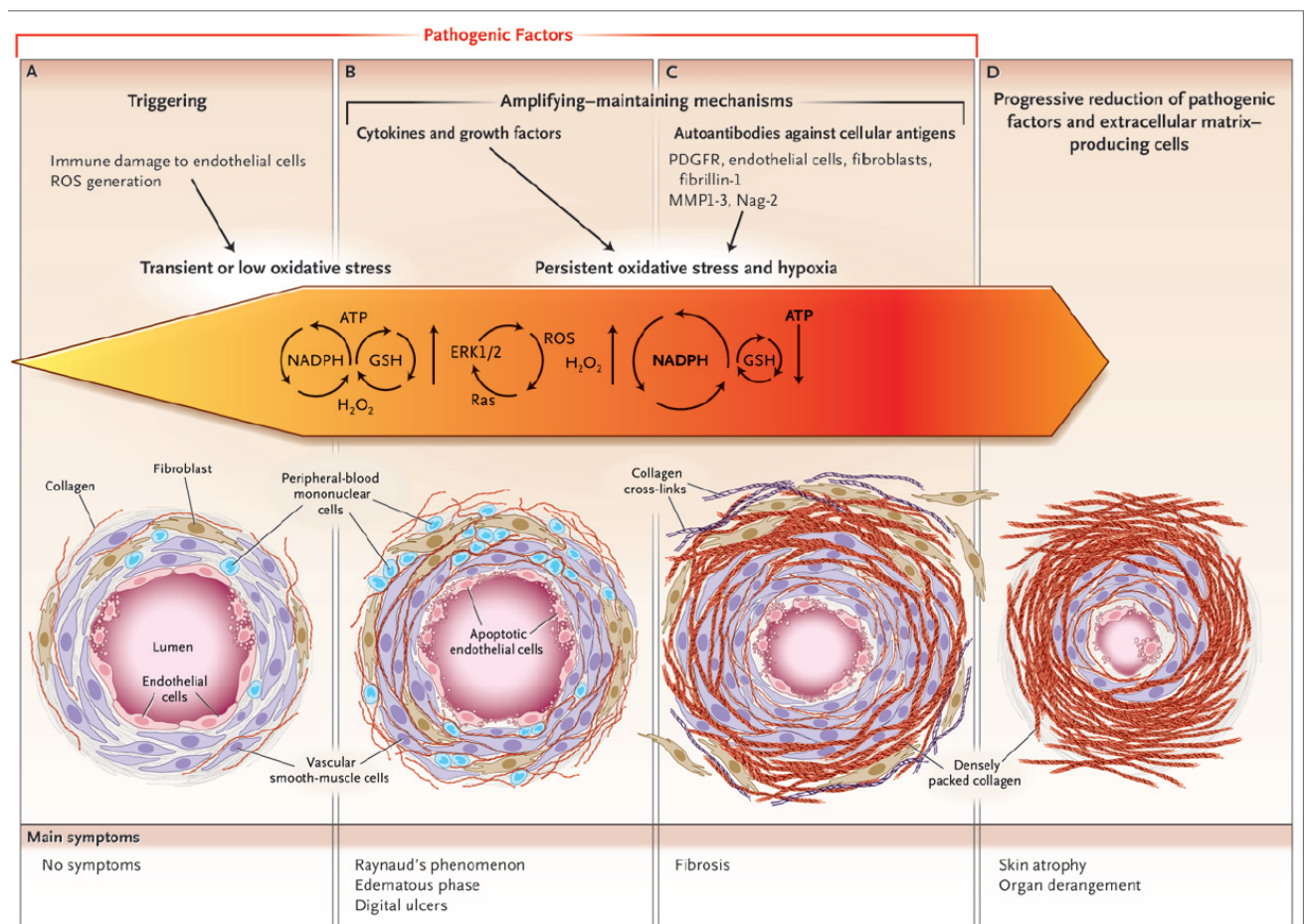


Figure 3 : Représentation schématique des principaux stades de l'angiogénèse. (Trojanowska) : 1 : A l'état quiescent, les cellules endothéliales ont de fins contacts entre elles et les péricytes. 2 : Le phénomène d'angiogénèse nécessite une dégradation locale des composants extracellulaires de la membrane basale par les protéases endothéliales, une perte des contacts cellules/cellules et cellules/matrice extra-cellulaire, une prolifération des cellules endothéliales, leur migration et la formation d'un germe de vaisseau non luminal. Les facteurs sécrétés par les cellules endothéliales attirent les péricytes et contribuent à la stabilisation des nouveaux vaisseaux. 3 : La maturation normale aboutit au rétablissement des contacts intercellulaires, la formation d'une lumière intime, la sécrétion d'éléments de la membrane basale et le recrutement de péricytes.

Les cellules endothéliales sont altérées, la membrane basale est épaissie et il existe des brèches endothéliales comme celles retrouvées dans l'athérome. D'un point de vue histologique, on peut observer au niveau des cellules endothéliales des mécanismes de perméabilité endothéliale, d'apoptose et de nécrose cellulaire. Dans la ScS, les lésions initiales semblent siéger au voisinage immédiat des cellules endothéliales. Les modifications endothéliales semblent précéder l'agrégation plaquettaire et l'infiltrat de cellules mononuclées. Les molécules d'adhésion auraient, à ce stade, un rôle majeur en favorisant le

recrutement de leucocytes activés dans les tissus cibles. Une apoptose précoce des cellules endothéliales a été mise en évidence. On observe également un infiltrat péri vasculaire de cellules mononuclées, un afflux de CD4+ ainsi qu'une prolifération intimale (myointimale) responsable à terme d'une fibrose et d'une obstruction des vaisseaux. Les liens entre cellules endothéliales et cellules musculaires lisses sont étroits avec une altération des cellules endothéliales en parallèle de l'évolution fibrosante (*Figure 4*). Le meilleur exemple d'altérations morphologiques des capillaires dans la SSc est la visualisation des capillaires unguéaux à la capillaroscopie avec les différents patterns décrits d'anomalies évolutives, tels que les ont décrits Maricq ou Cutolo (*Cutolo et al., 2007*) (*Thompson et al., 1984*).

Figure 4 : Les différents stades des lésions retrouvées dans la SSc (Gabielli et al., 2009)



1.1.3 Anomalies fonctionnelles de la vasculopathie

Les cellules endothéliales sont situées sur la couche interne des vaisseaux. Elles produisent des substances vasoactives qui régulent le tonus vasculaire, tels que des vasoconstricteurs (angiotensine II, thromboxane A₂ et l'endothéline-1(ET-1)) et des vasodilatateurs (oxyde nitrique et prostacyclines [PGI₂]). Les cellules endothéliales ont également une fonction barrière. Elles participent à de nombreuses interactions physiologiques telles que: la régulation de la coagulation, les réactions des réactions auto-immunes, les interactions avec les bactéries, les plaquettes et les leucocytes, la production d'une grande variété de molécules d'adhésion, de facteurs de croissance, de cytokines et de chemokines.

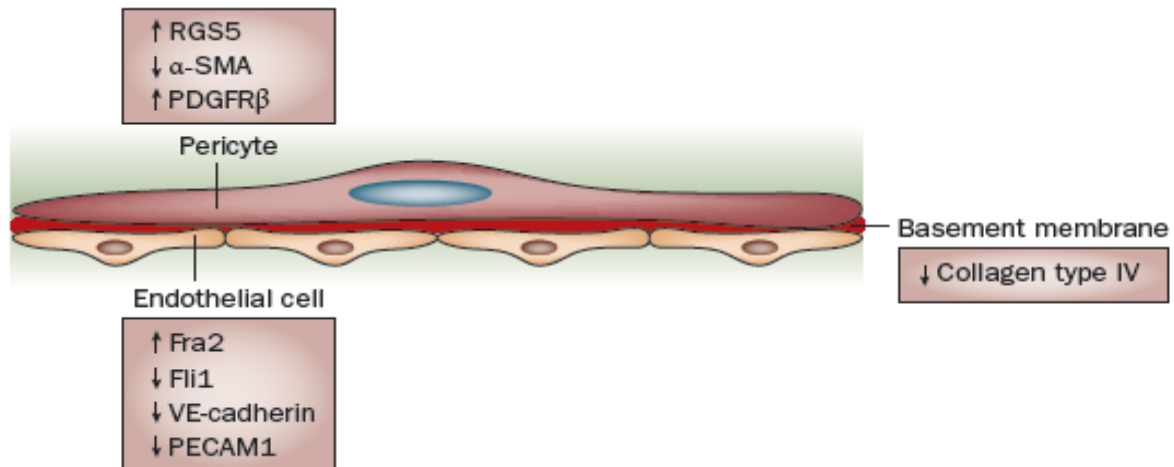


Figure 5 : Altérations moléculaires des composants vasculaires dans la ScS. Les péricytes expriment des marqueurs d'immaturité ou de prolifération, incluant la régulation positive de RGS5 et de PDGFR β et la régulation négative de α -SMA. Les cellules endothéliales sont caractérisées par un taux élevé du facteur de transcription pro apoptotique Fra2 et une diminution des taux du facteur de transcription Fli1, VE-cadherine et PECAM1 qui sont des indicateurs d'altération des contacts inter-cellulaires. Le taux de collagène IV est réduit, suggérant une altération de la membrane basale .(Trojanowska)

α -SMA. : α -smooth muscle actin, PDGFR β : platelet-derived growth factor β receptor, PECAM1, platelet endothelial cell adhesion molecule 1, RGS5, regulator of G protein signalling 5, VE-cadherine: vascular endothelial cadherin.

L'endothéline-1 (ET-1) est un vasoconstricteur puissant qui joue un rôle important dans la pathogénie de la ScS. Les taux plasmatiques d'ET-1 sont élevés dans cette pathologie, et l'ET-1 stimule la production de matrice extracellulaire par les fibroblastes, constituant ainsi un des liens entre les anomalies vasculaires et profibrotiques caractéristiques de la ScS. À l'opposé, le monoxyde d'azote d'origine endothéliale qui est vasodilatateur, antiagrégant et inhibiteur de la prolifération des cellules musculaires lisses vasculaires pourrait être déficitaire à l'état basal et produit en excès en présence de protéines de l'inflammation, participant aux phénomènes de cytotoxicité. Les modifications de production de vasomodulateurs favorisent le spasme vasculaire qui induit des lésions d'ischémie-reperfusion et la production de radicaux libres.

On observe au cours de la ScS une augmentation des marqueurs de peroxydation lipidique et d'oxydation protéique. Le clivage de certains auto-antigènes cibles d'auto-anticorps antitopoisomérase 1 et anti-ARN polymérase s'effectue en des sites spécifiques et cette réaction nécessite la fixation de différents métaux et la production de radicaux libres. La surproduction de radicaux pourrait provenir des phénomènes d'ischémie reperfusion liés au vasospasme. Une production accrue de radicaux libres par les fibroblastes dermiques de peau lésée a été associée à la synthèse augmentée de collagène.

En ce qui concerne les anomalies de l'angiogénèse, les concentrations sériques d'endostatine, inhibiteur de l'angiogénèse dérivé du collagène de type XVIII, sont élevées, et des élévations de VEGF et de la kallikreine ont été rapportées. L'augmentation de VEGF contraste avec l'existence chez les patients d'un déficit d'angiogénèse et un défaut de régulation de VEGF est suspecté.

Les cellules musculaires lisses participent au dérèglement du contrôle du tonus vasculaire. Des cellules secondairement activées sous l'effet de l'hypoxie et l'agrégation plaquettaire

(production de PDGF et TGF β) semblent migrer vers l'intima et se transformer en myofibroblastes, favorisant les lésions de prolifération myo-intimales observées au cours de la ScS. Les anomalies sur les fibroblastes ne seront pas abordées plus en détail.

1.1.4 Atteintes vasculaires cliniques périphériques de la ScS et prise en charge thérapeutique

Les traitements des atteintes microvasculaires cutanées dans la ScS sont limités, même si des avancées thérapeutiques sont arrivées ces dernières années avec notamment les thérapeutiques impliquées dans les anti-endothélines et les molécules impliquées dans l'oxide nitrique (NO). Dans le phénomène de Raynaud (RP), le traitement est d'abord préventif avec les mesures d'éviction au froid. Le traitement médical de référence médicale reste les inhibiteurs des canaux calciques. La nifédipine est la seule molécule commercialisée (avec l'indication phénomène de Raynaud) ayant fait l'objet d'études randomisées sérieuses (nifédipine versus placebo, nifédipine versus Iloprost intraveineuse, nifédipine versus losartan). Comparativement au

placebo, la nifédipine diminue significativement le nombre et la sévérité des crises. Néanmoins le bénéfice des inhibiteurs calciques dans le phénomène de Raynaud de la sclérodémie apparaît modeste (Thompson *et al.*, 2001). Le losartan est un antagoniste spécifique des récepteurs de type I de l'angiotensine II. Une étude pilote limitée en nombre de patients a montré une légère supériorité du losartan versus la nifédipine sur la sévérité des crises de Raynaud (Dziadzio *et al.*, 1999). L'iloprost (un analogue stable de la prostacycline) par voie orale (non disponible en Europe), à la dose de 50g ou 100g, versus placebo permet à 12 semaines une diminution de la durée et de la sévérité des crises, mais pas leur fréquence (Black *et al.*, 1998). L'iloprost utilisé par voie intraveineuse de manière séquentielle a montré dans une étude prospective, randomisée sur 12 mois, qu'il avait un effet légèrement supérieur à la nifédipine à la dose 40g/j per os (Scorza *et al.*, 2001). Le sildénafil, à la dose 50 mg/j, pourrait apporter un bénéfice sur le phénomène de Raynaud associé à la ScS (Lichtenstein, 2003), mais le niveau de preuve faible ne permet pas encore aujourd'hui d'utiliser ce médicament dans cette indication.

Le traitement des ulcérations digitales de la sclérodémie systémique est également d'abord préventif : éviter les activités à risque de blessure ou de coupure des extrémités, bonne hygiène cutanée et unguéale, protection des doigts, désinfection et soins locaux en cas de plaie. L'arrêt du tabac est un objectif majeur à obtenir chez les patients fumeurs qui sont quatre fois plus à risque d'ulcérations digitales (Harrison *et al.*, 2002). En cas d'ulcérations évolutives, une détersion mécanique est nécessaire, parfois chirurgicale. L'utilisation d'antibiotiques en topique est utile en cas de surinfection superficielle. Parfois une greffe cutanée est envisageable, mais les amputations phalangiennes ne peuvent pas toujours être évitées.

L'iloprost en intraveineux est recommandé en cure de cinq jours dans le phénomène de Raynaud sévère. Il a montré sa supériorité aux traitements conventionnels en cas d'ischémie digitale sévère ou de gangrène digitale. Dans une étude randomisée versus placebo, l'iloprost apportait un bénéfice en termes de fréquence de crises de Raynaud et de délai de cicatrisation des ulcérations (Wigley *et al.*, 1998) Utilisé dans une étude ouverte sous la forme d'une infusion par semaine toutes les six semaines sur une période de 12 mois, l'iloprost intraveineuse apporte un bénéfice sur le phénomène de Raynaud et pourrait avoir un effet sur l'évolution de la maladie comme en témoigne l'amélioration des scores cutanés (Scorza *et al.*, 2001). Une étude randomisée récente n'a pas retrouvé de bénéfice cutané. Le bosentan, premier antagoniste des récepteurs de l'endothéline, a démontré avec l'étude RAPIDS-2 qu'il réduisait significativement le risque de développement de nouveaux ulcères (Matucci-Cerinic

et al.). Cependant, le bosentan n'a pas montré de bénéfice sur la cicatrisation des ulcères évolutifs. Le bosentan a obtenu une AMM européenne pour la prévention des ulcérations digitales chez des patients atteints de ScS ayant des ulcères digitaux actifs. Des études sont en cours actuellement avec le sildénafil dans les ulcérations digitales (étude Seduce). La sympathectomie thoracique supérieure n'a souvent qu'un effet transitoire. La sympathectomie digitale est proposée par certains auteurs (Kotsis *et al.*, 2003)].

Dans d'autres cas les ulcérations digitales sont d'origine mécanique en regard des reliefs osseux ou des calcifications sous-cutanées. En cas de poussée inflammatoire en regard de calcifications, la colchicine peut avoir un effet anti-inflammatoire et antalgique intéressant (Fuchs *et al.*, 1986)]. La neurostimulation médullaire n'a plus que des indications exceptionnelles.

L'EULAR/EUSTAR a publié des recommandations de la prise en charge thérapeutique de la ScS récemment en 2011 (*figure 6*) (Walker *et al.*). Dans tous les cas, les traitements systémiques exposent à des effets secondaires ce qui laisse la place au développement potentiel d'un traitement local ou loco-régional.

Figure 6 : recommandation de l'EULAR/EUSTAR dans le traitement de la ScS (Walker et al.)

Table 1. Agreement with the EULAR/EUSTAR recommendations. Data are mean agreement (SD, range) and percent in the top 3 ratings of the survey scale (7-9).

Recommendations	Combined North America and Europe, n = 59		North America, n = 41		Europe, n = 18		p
SSc-related digital vasculopathy (RP, digital ulcers)							
1. Dihydropyridine-type calcium antagonists, usually oral nifedipine, should be considered for first-line therapy for SSc-RP, and intravenous iloprost, or other available intravenous prostanoids, for severe RP.	7.3 (1.6, 3-9)	74.5	7.2 (1.6, 4-9)	77.6	7.5 (1.6, 3-9)	72.2	0.673
2. Intravenous prostanoids (in particular iloprost) should be considered in the treatment of active digital ulcers in patients with SSc.	6.7 (2.5, 0-9)	67.8	6.1 (2.7, 0-9)	58.6	7.8 (1.8, 2-9)	88.9	0.001*
3. Bosentan should be considered in diffuse SSc with multiple digital ulcers after failure of calcium antagonists and, usually, prostanoid therapy.	5.8 (2.5, 0-9)	47.5	5.6 (2.4, 0-9)	43.9	6.3 (2.6, 1-9)	55.6	0.523
SSc-PAH							
4. Bosentan should be strongly considered to treat SSc-PAH.	7.7 (1.8, 3-9)	76.3	7.7 (1.8, 3-9)	83.0	7.5 (1.9, 4-9)	61.1	0.547
5. Sitaxentan may also be considered to treat SSc-PAH.	7.7 (1.8, 0-9)	83.1	7.5 (1.9, 0-9)	85.4	7.9 (1.5, 5-9)	77.8	0.445
6. Sildenafil may be considered to treat SSc-PAH.	8.1 (1.5, 2-9)	89.8	8.1 (1.5, 2-9)	95.1	8.1 (1.5, 4-9)	89.0	0.696
7. Intravenous epoprostenol should be considered for the treatment of patients with severe SSc-PAH.	7.8 (2.0, 0-9)	83.1	8.5 (1, 5-9)	95.0	6.5 (2.7, 0-9)	55.5	0.006*
SSc-related skin involvement							
8. Methotrexate may be considered for treatment of skin manifestations of early diffuse SSc.	5.3 (2.8, 0-9)	40.3	5.2 (3, 0-9)	41.1	5.5 (2.6, 0-9)	38.9	0.962
SSc-ILD							
9. Cyclophosphamide should be considered for treatment of SSc-ILD.	7.7 (1.4, 1-9)	84.7	7.7 (1.5, 1-9)	87.6	7.7 (1.2, 6-9)	77.8	0.755
SSc-SRC							
10. ACE inhibitors should be used in the treatment of SRC.	8.9 (0.6, 5-9)	98.3	8.9 (0.3, 9)	100	8.8 (0.9, 5-9)	94.5	0.187
11. Patients on steroids should be carefully monitored for blood pressure and renal function.	8.6 (1.0, 4-9)	96.7	8.6 (1.1, 4-9)	95.2	8.6 (0.7, 7-9)	100	0.626
SSc-related gastrointestinal disease							
12. PPI should be used for the prevention of SSc-related gastroesophageal reflux, esophageal ulcers and strictures.**	8.3 (1.5, 2-9)	89.9	8.2 (1.7, 2-9)	87.9	8.5 (0.8, 6-9)	94.5	0.518
13. Prokinetic drugs should be used for the management of SSc-related symptomatic motility disturbances (dysphagia, GERD, early satiety, bloating, pseudo-obstruction, etc.).	7.1 (1.9, 0-9)	71.1	7.1 (1.9, 1-9)	70.8	7.1 (2.0, 0-9)	72.3	0.828
14. When malabsorption is caused by bacterial overgrowth, rotating antibiotics may be useful in SSc patients.	8.3 (1.1, 4-9)	93.3	8.4 (1.1, 4-9)	95.2	8.1 (1.1, 6-9)	88.9	0.585

* Mean North American rheumatologist agreement vs European rheumatologist agreement that was statistically different ($p < 0.05$). ** Recommendations that had significantly different agreement ($p < 0.05$) by respondents who were EULAR/EUSTAR recommendation authors vs respondents who were not EULAR/EUSTAR recommendation authors. EULAR: European League Against Rheumatism; EUSTAR: EULAR Scleroderma Trials and Research group; SSc: systemic sclerosis; RP: Raynaud's phenomenon; PAH: pulmonary arterial hypertension; ILD: interstitial lung disease; SRC: scleroderma renal crisis; ACE: angiotensin-converting enzyme; PPI: proton pump inhibitors; GERD: gastroesophageal reflux disease.

1.2 Principe de la iontophorèse

1.2.1 Principes de Base

La iontophorèse est connue comme un test physiologique en microcirculation cutanée. C'est un dispositif qui permet la diffusion de solutions à travers la peau grâce à une stimulation électrique d'une façon non invasive. Le courant administré peut être réglable en intensité et en sens (anodal ou cathodal). Les molécules les plus utilisées en test physiologique sont l'acétylcholine et le nitroprussiate de sodium (SNP). Ces tests physiologiques sont généralement réalisés avec de faibles intensités et pendant un temps de courte durée (quelques secondes à quelques minutes). La iontophorèse à l'acétylcholine est utilisée comme un test endothélium dépendant et la iontophorèse au SNP un test non endothélium dépendant. La diffusion transcutanée des molécules via la iontophorèse est basé sur deux principes que sont l'électrorépulsion et l'électroosmose.

L'électrorépulsion est la migration d'une molécule ionisée par répulsion des charges du même signe. Il est donc important d'estimer la fraction ionisée et surtout la polarité des molécules utilisées afin d'estimer au mieux le sens du courant permettant une diffusion transcutanée. Ainsi, si une substance est chargée positivement, elle diffusera à travers la peau au niveau de l'anode (+) selon le principe de l'électrorépulsion des charges (*figure 7*).

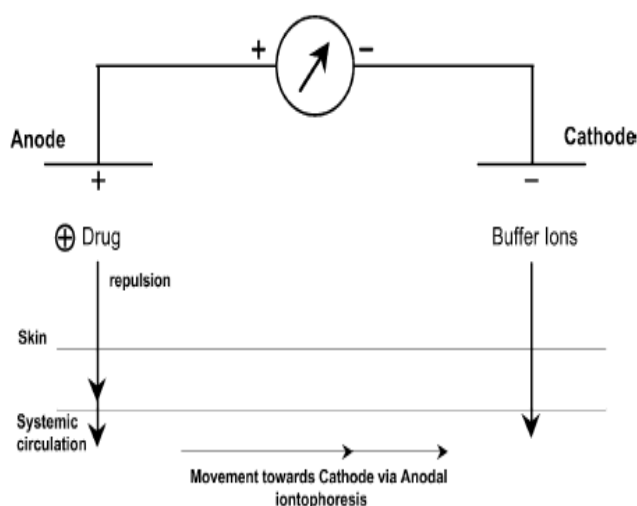


Figure 7: La technique de la iontophorèse et le principe de l'électrorépulsion. Le courant appliqué sur une molécule chargée positivement (cation) migrera de l'anode vers la cathode. Les cations de la molécule passent à travers la peau et sont éventuellement absorbés dans la circulation systémique (Wang et al., 2005).

L'électroosmose est la migration d'une molécule (même non ionisée) par entraînement liée au flux d'eau de l'anode vers la cathode pendant la iontophorèse. La migration est notamment due aux ions Na^+ et à la charge négative de la peau. Sous l'effet d'un courant continu, un solvant peut entraîner avec lui, lors de sa migration des substances dissoutes.

Chaque ion a une valeur de transport (t) qui correspond à la fraction de courant total transféré par l'ion et qui est variable selon les propriétés physicochimique des ions. t_{Na^+} est supérieur à t_{Cl^-} ce qui explique que la migration des ions Na^+ est facilitée (augmentation dans le compartiment cathodal). Du fait de ce gradient physicochimique, le flux osmotique de l'eau est induit de l'anode vers la cathode (Wang et al., 2005)

Cet effet est pH-dépendant. Ainsi, dans des conditions physiologiques, la peau a une charge négative ce qui facilite le transport de molécules cationiques. Au cours de ce transport, le solvant entraîné pourrait à son tour permettre la diffusion de molécules dissoutes en son sein.

D'autres mécanismes seraient impliqués :

Le mécanisme de transfert par « shunt » : ce mécanisme découle de l'observation que la diffusion transdermique de molécules est favorisée par la présence de follicules pileux, de

glandes sébacées ou d'imperfections de la peau. Par exemple, les follicules pileux représenteraient un shunt permettant un transport plus profond de molécules à travers la peau (Hirvonen *et al.*, 1998).

Le mécanisme par transfert par « flip-flop » : cette théorie repose sur le principe que la perméabilité cutanée peut-être modifiée sous l'effet d'un courant électrique. Les polypeptides des membranes cellulaires s'orienteraient alors parallèlement, permettant la création de pores voltage-dépendants. Ce mécanisme de pénétration transcellulaire a été visualisé après iontophorèse en microscopie électronique (Monteiro-Riviere *et al.*, 1994).

1.2.2 Facteurs influençant l'absorption en iontophorèse

Du fait des principes exposés et notamment de l'électrorépulsion, les molécules hydrophiles et de faible poids moléculaire sont les meilleures candidates à la iontophorèse transdermique. Néanmoins, de nombreux facteurs sont impliqués dans l'absorption des molécules (Kalia *et al.*, 2004). La pénétration par iontophorèse est inversement dépendante du poids moléculaire, mais les autres mécanismes impliqués dans la iontophorèse telle que l'électroosmose font que des molécules neutres et de haut poids moléculaire telles que les protéines ont pu être transférées en transcutané via ce procédé. Les facteurs principaux impliqués sont :

1.2.2.1 ceux liés à la molécule choisie :

- pH et pKa de la molécule

L'effet du pH est important, car il conditionne la polarité de la molécule. Le passage en iontophorèse est en théorie optimal pour un pH inférieur à 4 ou supérieur à 7 pour maintenir un taux d'ionisation de la molécule important en fonction de son pKa. Par exemple, pour une base faible, le taux de ionisation va être très faible à un pH au dessus de son pKa et ne va pas permettre une électromigration (mais la diffusion peut éventuellement être induite par le phénomène d'électroosmose).

Dans les conditions physiologiques, la peau est chargée négativement avec un pH cutané proche de 4. Les molécules chargées positivement auront donc un passage facilité en iontophorèse cutanée. D'autres mécanismes sont dépendants du pH. Par exemple, lorsque le pH décroît, la concentration en ions hydrogènes augmente et une vasodilatation est initiée liée à l'activation des fibres nociceptives C (réflexe axonal non lié à l'effet de la molécule mais liée au relargage de la substance P). L'important serait alors de réussir à se situer à un pH proche de 7, surtout lorsque l'on travaille avec des molécules vasodilatatrices. A pH trop bas, la pénétration des ions hydrogènes prend le dessus.

- la compétition ionique :

La migration ionique est fortement liée au courant de charge opposée qui est appliquée. Il peut exister des ions de même charge dans la solution appliquée (par exemple dans les excipients). Ces ions sont appelés co-ions et peuvent devenir compétitifs avec les ions du principe actif choisi.

- le gradient de concentration :

Il existe en théorie un lien entre la concentration de la molécule et la quantité de molécule diffusée en transcutanée. En pratique, ce lien n'est pas toujours constaté du fait de la possibilité d'un effet plateau, par saturation du « transport iontophorétique ».

- le coefficient de diffusion :

Il décroît avec la taille de la molécule (chargée ioniquement ou non)

- la taille de la molécule :

Elle implique un coefficient de perméabilité variable. Des protéines même avec des poids moléculaires élevés (comme l'insuline) ont pu néanmoins être délivrées en iontophorèse du fait du phénomène d'électroosmose.

- La solubilité de la molécule

Bien entendu, le critère de seuil de solubilité de la molécule sélectionnée est capital. Le solvant ou l'excipient utilisé est également influent puisqu'il peut influencer le pH de la solution et interférer en tant que co-ion.

Afin d'optimiser la passage d'une molécule en transcutané, deux stratégies peuvent se dégager : tenter de modifier chimiquement la molécule et/ou son activité thermodynamique (Wang *et al.*, 2005). Les modifications chimiques permettent de modifier l'organisation de la structure lipidique intercellulaire, la fluidité lipidique, l'altération des protéines cellulaires et dans certains cas l'extraction lipidique intercellulaire. Les résultats d'optimisation de la molécule pour pénétrer en transcutané via la iontophorèse sont limités, notamment pour les molécules hydrophiles. Certaines études ont par exemple montré l'amélioration de passage de molécules en présence d'acides gras tel que l'acide oléique pour des molécules cationiques du fait de l'amélioration de leur lipophilicité in vitro sur la peau du rat (salbutamol, nicotine, diltiazem hydrochloride) (Nolan *et al.*, 2007).

Les propriétés thermodynamiques éventuellement développées sont liées à l'utilisation de techniques du type électroporation (technique de création de pores dans les membranes cellulaires par chocs électriques brefs, inférieur à 1 seconde, et intenses (50 à 500 Volt)), ou de type sonophorèse (diffusion de molécules facilitée par les ultra sons), qui ne seront pas développées ici. Certaines études ont testé leur association avec la iontophorèse. Un pré-traitement avec une basse fréquence d'ultra sons (0.2 W :cm² pendant 2 heures) n'augmente pas la diffusion du sodium nonivamide acetate mais la combinaison de la iontophorèse (0.5 mA/cm²) avec la sonophorèse augmente la diffusion transdermique versus la iontophorèse seule (Fang *et al.*, 2002).

1.2.2.2 ceux liés à la iontophorèse :

- un courant continu ou pulsé : L'application d'un courant continu de manière prolongée peut modifier la polarité cutanée et de ce fait l'efficacité de la iontophorèse (proportionnellement à la longueur d'application du courant). L'administration d'un courant de manière pulsée permet pendant les périodes « OFF » que la peau retrouve sa polarité initiale. L'effet néanmoins de ce retour à l'état initial diminue si la fréquence du courant pulsé augmente.

- le sens du courant / charge : le choix du sens du courant dépendra de la polarité de la molécule ionisée.

- la quantité de courant et la densité de courant

La dose qui diffuse dépend de la quantité de courant. $Q = I \times t$ avec Q=quantité de courant en coulombs, I= intensité en ampère et t= temps en seconde.

La dose de courant administrée est également limitée par la taille de l'électrode. C'est le facteur densité de courant qui est alors important. On avance une densité acceptable jusqu'à 500 mA/ cm². Au-delà, l'effet expose à l'inconfort du patient et des risques de brûlures.

1.2.2.3 ceux liés aux propriétés cutanées:

- épaisseur cutanée:

L'effet de la iontophorèse serait variable selon l'état de la peau (présence de peau glabre ou non, sexe féminin ou non...). Il est également variable selon l'épaisseur de l'épiderme. L'épaisseur de l'épiderme est également un facteur influençant la iontophorèse. Cette épaisseur peut varier de 0.06 mm pour les paupières à 0.8 mm pour la paume ou la plante des pieds. La principale limite de l'épiderme étant la couche/barrière superficielle de l'épiderme, le stratum cornéum (10 à 20 μm d'épaisseur). La fonction première de cette couche étant de protéger la peau de la perte en eau et des agents chimiques extérieurs, la iontophorèse doit donc contrecarrer les propriétés de fonction barrière du stratum cornéum.

L'état d'« irritation » cutanée ou d'hydratation modifie les propriétés cutanées et donc le phénomène de migration via la iontophorèse. Avant l'application de l'électrode de iontophorèse, il est conseillé d'appliquer de l'eau désionisée ou de l'alcool à 70° afin d'homogénéiser le support cutané et de limiter des localisations de micro brûlures.

A des valeurs de pH au dessus de 4, la peau est chargée négativement, impliquant que les charges positives (telles que le Na^+) soient transportées plus facilement afin de neutraliser la charge et de maintenir une électroneutralité (Wang *et al.*, 2005)

- densité microvasculaire locale :

Par définition, la microcirculation est limitée à des vaisseaux plus petits que 100 μm (par exemple artérioles, capillaires et veinules). Le derme est la structure anatomique cutanée qui est composée des éléments microvasculaires. Le réseau vasculaire consiste en un plexus superficiel subpapillaire et un réseau plus profond appelé plexus cutané (figure 8).

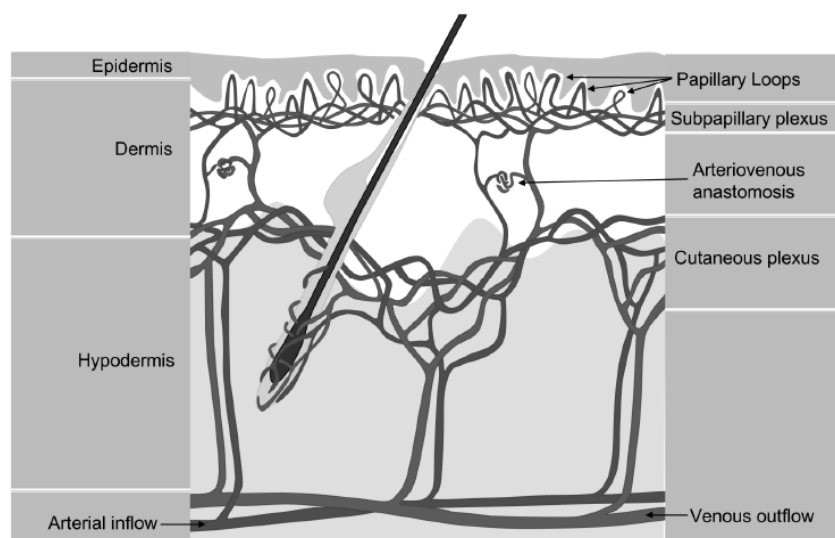


Figure 8 : anatomie schématique vasculaire cutanée (issue de la thèse de Fernando Morales. *Improving the clinical applicability of laser Doppler perfusion monitoring* 2005 Electronic version ISBN: 90-367-417-1©)

Le plexus subpapillaire est situé à une profondeur de 400-500 μm par rapport à la surface. Les anses capillaires se terminent par des anses papillaires (10 μm de diamètre) au niveau de la basale de l'épiderme. Le plexus subpapillaire est connecté au plexus cutané localisé à environ 1.9 mm de la surface de la peau par les artérioles ascendantes et les veinules descendantes. Sur certaines localisations anatomiques tels que les doigts, le nez, les lèvres et les oreilles, il existe des interconnexions entre les artérioles et les veinules, assurées par des shunts vasculaires, appelés anastomoses artério-veineuses, impliquées notamment dans l'action de thermorégulation. La densité du réseau microvasculaire influence l'effet de la iontophorèse

avec une action attendue supérieure en cas de faible densité vasculaire du fait probablement d'une clairance élevée.

Il existe également un réseau vasculaire dans l'hypoderme. La plupart du flux dans cette couche cutanée est composé de paires d'artéριοles ascendantes et de veinules descendantes. Quelques structures particulières (follicules pileux et gland) sont surmontées d'un petit réseau capillaire. Le flux microcirculatoire change selon les variations hormonales, physiques (température et pression) et est contrôlé par le système nerveux autonome via des variations de flux au niveau des artéριοles.

En physiologie, l'effet de la iontophorèse va surtout être fonction de la densité capillaire. Lors de l'utilisation de la iontophorèse en thérapeutique, l'effet de la iontophorèse sera fonction de la densité mais également de la fonctionnalité des vaisseaux.

-Résistance cutanée :

La résistance électrique est proportionnelle à la longueur du conducteur. Au niveau cutané, le calcul de la résistance doit donc être réalisé toujours avec la même distance entre les électrodes. $U = RI$ avec $U =$ quantité de courant délivrée, $R =$ résistance et $I =$ intensité en ampère donc si R varie pour une intensité imposée, le voltage résultant varie également. La résistance a été étudiée dans les études INFLUX rat et INFLUX IT VS. Elle a été étudiée en standardisant la distance entre les électrodes passives et actives de la iontophorèse. Il est à noter que la résistance cutanée peut être modifiée selon le protocole de iontophorèse élaboré (notamment courant continu ou en switching, c'est-à-dire alternance rapide de la polarité du courant) et également par d'autres facteurs telle que l'hydratation cutanée notamment. Ces deux facteurs sont d'ailleurs peut-être liés. La résistance cutanée diminue lors de iontophorèse en switching, probablement du fait de la diminution de l'hydratation cutanée (Okuno *et al.*, 2008).

1.2.3 La iontophorèse : une alternative à certains traitements oraux ?

La connaissance des quantités de principe actif des molécules diffusées via la iontophorèse dans les différents compartiments (intra dermique et circulation systémique notamment) est limitée. Elle est bien sûr fonction de la densité capillaire et probablement de la fonctionnalité capillaire intra dermique. Les moyens pour estimer la diffusion et la cinétique d'une molécule en intra dermique sont limités (cf partie sur les moyens d'exploration de la microcirculation). Néanmoins, lorsqu'une molécule est administrée en intra dermique, la concentration interstitielle de la molécule décroît exponentiellement au fil du temps en fonction du flux sanguin cutané. Par exemple, un flux élevé (par exemple sur le dos du doigt) va accroître la clairance cutanée (Cracowski *et al.*). C'est cette hypothèse qui peut expliquer pourquoi la iontophorèse de nitroprussiate de sodium (SNP) conduit à une vasodilatation chez le volontaire sain sur l'avant-bras mais pas sur le dos du doigt (Roustit *et al.*, 2009). A ce concept sont rajoutés plusieurs autres phénomènes complexes comme le fait que la clairance cutanée d'une molécule va diminuer lorsque le flux sanguin local est réduit par l'injection concomitante d'une autre molécule.

L'effet de la iontophorèse va surtout être une action topique préférentielle (locale ou loco-régionale) mais également systémique. L'effet systémique de la iontophorèse a déjà été utilisé en thérapeutique. L'avantage alors de la iontophorèse est de shunter le premier passage hépatique et le métabolisme hépatique. Cette technique permet alors de ne pas retrouver les inconvénients de la même molécule administrée en parentérale (ex : lidocaïne hydrochloride et epinephrine en patch de iontophorèse : Lidosite®) ou des variations de cette même molécule liées aux variations d'une absorption per os.

1.2.4 Les effets secondaires de la iontophorèse

Les effets secondaires majeurs de la iontophorèse sont peu nombreux. Des études ont été réalisées sur l'effet de la iontophorèse sur l'intégrité de la peau avec de l'impédance spectroscopie, la microscopie, la calorimétrie avec scanner différentiel, la spectrométrie infra rouge, la perte en eau transépithéliale, des scores visuels et l'évaluation des patients et ont montré dans une étude en simple aveugle comparant la iontophorèse seule (3 heures à 250 $\mu\text{A}/\text{cm}^2$ à une iontophorèse avec un surfactant en prétraitement. Les principaux effets étaient les paresthésies et le prurit pendant les 30 premières minutes. La iontophorèse associée au surfactant augmentait la perte d'eau transépithéliale ainsi que l'érythème cutané. Elle augmentait également le risque d'œdème en comparaison de la iontophorèse seule. Le surfactant en pré traitement induisait plus d'irritation cutanée (érythème et œdème) sans altérer la fonctionnalité de la barrière cutanée. (Li *et al.*, 2005) La méthodologie est présentée comme sécurisée (Kumar *et al.*, 2008). La plupart des effets secondaires sont mineurs avec des fourmillements, des prurits, des érythèmes et des oedèmes. Des cas de brûlures ont été rapportés (Li *et al.*, 2005). Elles sont liées à l'intensité, au courant continu et seraient plus fréquentes à la cathode (pH plus élevé). Des cas ont été rapportés de « Galvanic urticaria » (Meffert, 1999) (Lacy *et al.*, 2006; Li *et al.*, 2005). Des dyspigmentations ont également été rapportées, dont le risque serait proportionnel au temps d'exposition à la iontophorèse. Le nombre d'effets secondaires est fonction de l'exposition au courant (en termes de charge et densité de courant délivré). Des systèmes de délivrance de iontophorèse en combinaison avec un pré-traitement par un surfactant n'ont pas montré moins d'effets secondaires cutanés (Li *et al.*, 2005). Il existe également quelques cas décrits de sensibilisation de contact aux molécules appliquées (kétoprofène), aux composants des électrodes et aux gels des électrodes (propylène glycol) ou au nickel.

Il faut également respecter les contre-indications de la iontophorèse qui sont la présence de dispositifs implantables tels que les pace-makers ou une hypersensibilité à la molécule appliquée.

1.2.5 La iontophorèse thérapeutique

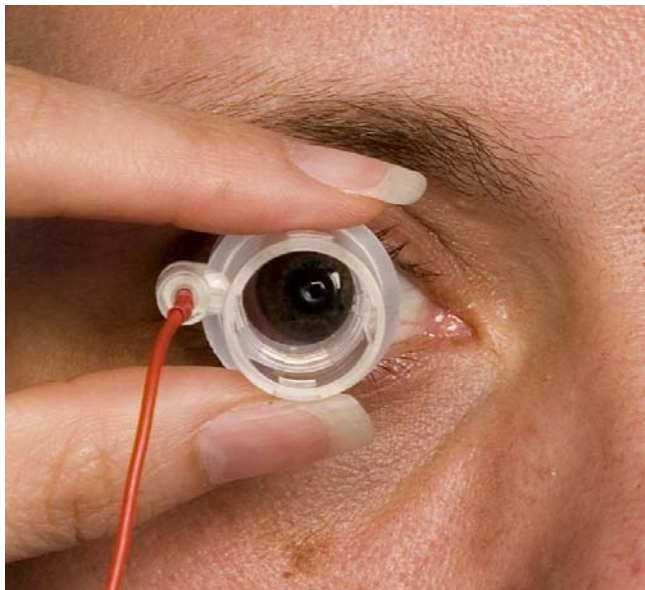
Seuls quelques dispositifs ont été commercialisés et peuvent être considérés comme de la iontophorèse thérapeutique.

- La iontophorèse sans principe actif spécifique est utilisée actuellement en dermatologie dans le traitement de l'hyperhydrose. Les mécanismes impliqués ne sont pas clairement compris, mais reposeraient sur le fait que la iontophorèse engendrerait une hyperkératose des pores des glandes sébacées puis leur obstruction. D'autres auteurs évoqueraient une implication du gradient électrochimique des sécrétions sébacées et un mécanisme de rétrocontrôle (Wang *et al.*, 2005). En France, l'appareil commercialisé est I2M-A (I2M, Caen, France). L'intensité développée par l'appareil est fonction de la résistance de la peau (de l'ordre de 5000 ohms) variable d'un sujet à un autre suivant le sexe, l'âge et le degré d'humidité et d'hydratation. L'appareil est réglé pour ne pouvoir délivrer une intensité supérieure à 30 mA (20 mA étant considérée comme l'intensité souhaitée pour une efficacité optimale du principe).
- Un dispositif d'électrostimulation a été développé dans les plaies chroniques, notamment en phase de déterision et de bourgeonnement. L'appareil Woundel® (DTF, Saint Etienne, France) est actuellement commercialisé en France et repose sur l'utilisation d'un courant continu pulsé de fréquence 125 Hz générant un champ électrique proche des champs

électriques endogènes de la peau et l'utilisation d'électrodes pansements spécifiques permettant le dispersion du courant pulsé et le maintien du milieu humide de la plaie. Le rationnel de l'efficacité du Woundel® reposerait sur le fait qu'une plaie interromprait la continuité de la peau et ferait un « court-circuit » des potentiels aboutissant à une fuite du courant électrique. (Junger *et al.*, 2008). Les charges électriques peuvent schématiquement parvenir dans les cellules de deux manières : par le biais de canaux ioniques et par le biais de l'action capacitive de la membrane cellulaire. La transmission des charges électriques se fait par les ions Na^+ , K^+ , Ca^{2+} ou Cl^- . L'application d'un champ électrique sur une plaie déclencherait de multiples comportements cellulaires à type de :

- Synthèse protéique : augmentation de la synthèse d'ADN et de collagène, élévation en ATP dans la peau et absorption accrue d'acides aminés.
 - Migration cellulaire : migration préférentielle des macrophages ou des fibroblastes ou des kératinocytes vers la cathode.
 - Réagencement cellulaire: réorientation, élongation et migration directe. Dans l'angiogénèse, les myéloblastes et les cellules endothéliales se dresseraient dans leur axe longitudinal perpendiculairement au vecteur du champ électrique. Certains des mécanismes à l'origine de ces phénomènes sont connus. Les cellules vasculo-endothéliales soumises à un champ électrique ont un effet multiplicateur sur la sécrétion du facteur de croissance Vascular Endothelial Growth Factor (VEGF) dans un délai de 5 minutes. Un premier pic serait atteint en 30 minutes, le second entre 4 et 24 heures. L'autosécrétion du ligand VEGF et l'activation de VEGFR1 sont des éléments fondamentaux de la transmission de cette réaction.
- La iontophorèse a été utilisée en ophtalmologie par voie transconjonctivale, transcornéenne ou transclérale (*figure 9*). Les mécanismes de passage des produits pharmacologiques ne sont pas connus. La iontophorèse pourrait trouver de nombreuses applications thérapeutiques en ophtalmologie dans le traitement des inflammations et des infections du segment postérieur en alternative ou complément des injections intra vitréennes, sous conjonctivales ou latéro-bulbaires ou d'un traitement systémique. Une autre application pourrait être l'administration de facteurs anti angiogéniques vers la rétine ou la choroïde. La spécificité des différents milieux anatomiques ophtalmologiques ne rend pas transposable ces données à la peau.

Figure 9 : appareil de iontophorèse par voie ophtalmique : EyeGate® II system (Eygate Pharmaceuticals, Inc., Waltham, USA)



Les applications commerciales de iontophorèse thérapeutique sont rares. Nous pouvons citer Ionsys® qui était le premier un dispositif iontophorétique avec administration transdermique de fentanyl contrôlé par le patient pour le contrôle de la douleur aiguë post-opératoire (Mayes *et al.*, 2006). Il permettait d'éviter les limitations de l'administration en intra veineux, notamment d'éviter les effets indésirables de saignement, d'infiltration du cathéter intraveineux et de dysfonctionnement de pompe. L'intérêt du système était de potentialiser l'administration systémique d'une molécule de haut poids moléculaire et de pouvoir effectuer une délivrance de la molécule contrôlée par le patient et avec un minimum de variabilité inter-sujet. La commercialisation de l'Ionsys® dans les pays membres de la communauté européenne a été approuvée par la Commission Européenne pour l'usage intra-hospitalier en janvier 2006. En septembre 2008, Janssen-Cilag a retiré le dispositif du marché par mesure de précaution suite à la découverte de traces de corrosion sur un composant du système dans un des lots du produit. Cette corrosion est susceptible d'entraîner l'auto-activation du système d'administration du fentanyl et la libération de doses non prescrites au patient. En conséquence, IONSYS n'est actuellement disponible dans aucun pays de l'UE. Aucun effet indésirable grave n'a été rapporté suite à la découverte de ce dysfonctionnement. Le dispositif serait actuellement réétudié pour recommercialisation. L'autre produit commercialisé est le Lidosite® (Vysteris Inc, USA) (Dixit *et al.*, 2007) pour le traitement de la douleur locale. Il s'agit de la lidocaïne hydrochloride associée à de l'épinéphrine combinées dans un patch commercialisé aux USA. Un troisième produit pourrait être développé dans les migraines avec vomissement. Il s'agit d'une iontophorèse avec du sumatriptan (Zeltrix®).

1.3 Les méthodes d'explorations de la microcirculation

Les fonctions de l'endothélium vasculaire incluent le contrôle du tonus vasculaire, l'inhibition de l'agrégation plaquettaire, la régulation des attachements pariétaux des leucocytes, leur enroulement et leur transmigration, la régulation de la prolifération des cellules musculaires lisses, et la modulation de la perméabilité du mur vasculaire.

La plupart du temps, le terme de « fonction endothéliale » fait référence en sa capacité à relarguer des composants qui peuvent induire une relaxation directe sur les cellules musculaires lisses sans le mur vasculaire que sont le NO, la prostacycline et les facteurs hyperpolarisants d'origine endothéliale (endothelium-derived hyperpolarizing factors, EDHFs). Les différents outils pour étudier la microcirculation sont :

1.3.1 La capillaroscopie :

C'est la vidéocapillaroscopie qui est actuellement la méthode utilisée en pratique courante clinique, notamment dans la prise en charge des phénomènes de Raynaud (RP) secondaires. La capillaroscopie est un moyen simple et non invasif d'explorer l'atteinte microvasculaire. Elle permet d'évaluer la morphologie des capillaires cutanés unguéaux (*figure 10*). Des aspects descriptifs évocateurs sont des critères diagnostiques de certaines pathologies, notamment la ScS (*figure 11*). La description capillaroscopique permet également de typer les profils capillaroscopiques en « pattern » qui sont corrélés à l'évolution de la maladie ScS.

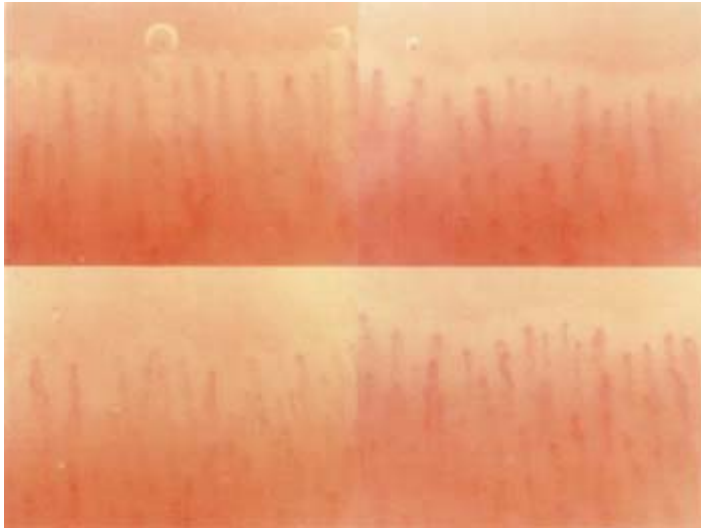


Figure 9: capillaroscopie normale (grossissement x50)

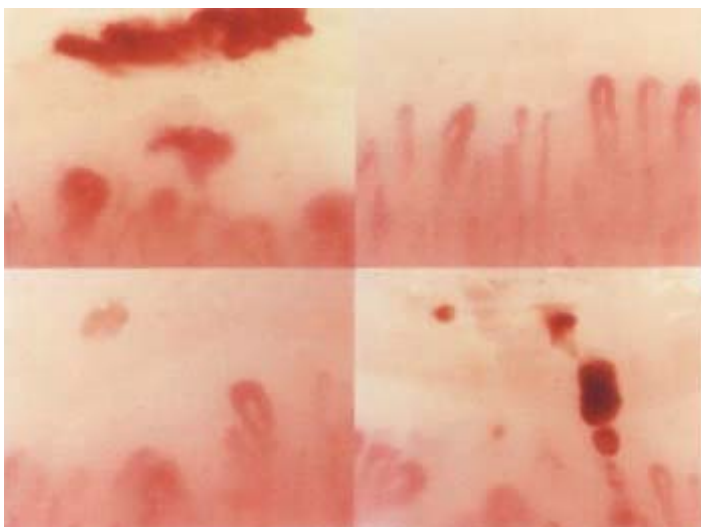


Figure 10 : capillaroscopie anormale avec mégacapillaires (en haut à gauche) et zones hémorragiques (en bas à droite) (grossissement x50)

1.3.2 La fluxmétrie Laser Doppler (LDF):

La technique Laser Doppler est basée sur la réflexion d'un faisceau lumière monochromatique (**principe du laser**). La lumière subit des changements de longueur d'onde (**effet Doppler**) quand elle heurte des structures en mouvement, notamment les cellules sanguines. L'amplitude et la distribution des fréquences de ces changements de longueur d'onde sont liées au nombre et à la vitesse des cellules sanguines, principalement les globules rouges. Plusieurs signaux différents peuvent être enregistrés, mais le flux sanguin de globules rouges (soit le produit de la vitesse et la concentration de globules rouges dans le volume de mesure) est le plus souvent utilisé. Actuellement, la majorité des lasers sont équipés de diodes laser permettant d'émettre un faisceau de longueur d'onde 780 nm, qui offrent:

- une bonne pénétration permettant de s'affranchir de la couleur de la peau
- correspond à l'absorption de l'hémoglobine oxydée et non oxygénée, permettant d'éliminer la dépendance par rapport à la saturation en oxygène (Zijlstra *et al.*, 1991)

Des filtres passe-haut du 3^{ème} ordre ayant une fréquence de coupure de -3dB à 20 Hz extraient la partie fluctuante du signal de sortie total, incluant les composantes Doppler. Ce signal est ensuite amplifié et normalisé pour être indépendant de l'intensité lumineuse. Une relation linéaire entre le signal de fluxmétrie laser Doppler et le débit microvasculaire a été démontrée pour des valeurs de perfusion allant de 0 à plus de 300 ml/min/100 g (Ahn *et al.*, 1987).

La technique de **fluxmétrie Laser Doppler (LDF)** permet d'évaluer in vivo le flux sanguin cutané au repos ou lors de tests physiologiques ou fonctionnels de manière non invasive. Il consiste à appliquer sur la peau un faisceau optique conducteur de la lumière associé au récepteur, avec un volume d'étude de l'ordre d'une $\frac{1}{2}$ sphère d'un mm de diamètre. Il émet un faisceau hélium-néon monochromatique d'une longueur d'onde de 780 nm de faible puissance (2 mW) en continu (*figure 11*).



Figure 11 : de gauche à droite : sonde intégrée de LDF comportant 7 émetteurs et 7 récepteurs, sonde simple fibre couplée à un élément chauffant, sonde de iontophorèse de LDF, couplée à un élément chauffant.

Les avantages de la technique sont celle d'une méthode non invasive, une grande résolution temporelle (avec une acquisition de signal très fréquente (32 Hz)) car la mesure de la perfusion cutanée est continue avec un temps de réponse rapide (inférieur à 0.1 seconde). Un des principaux inconvénients est la mauvaise résolution spatiale surtout avec les sondes ne comportant qu'une seule fibre émettrice/réceptrice (*figure 11*, au milieu, le volume exploré est identique à la surface de la sonde soit 1 mm^3).

Cette méthode est donc adaptée à des variations vasculaires rapides telles qu'elles sont obtenues lors de test microcirculatoire comme lors de l'hyperhémie post occlusive (PORH). Nous l'avons utilisé pour l'étude ancillaire d'INFLUX-VS (effet du sildénafil sur la PORH chez le volontaire sain). (*Figure 12*)

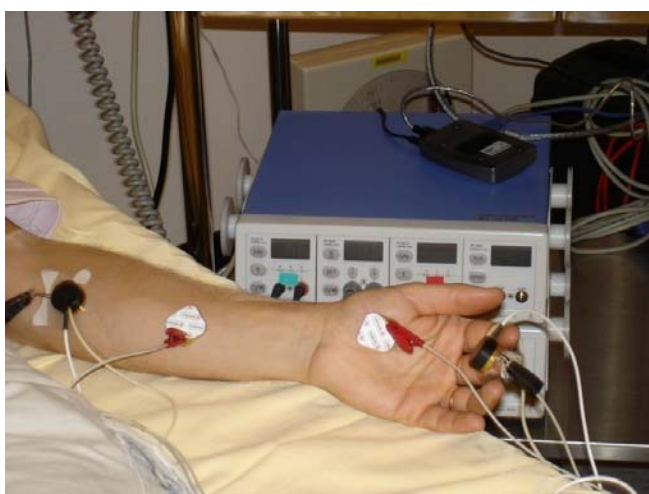


Figure 12 : LDF avec sonde de iontophorèse au niveau du tiers supérieur de l'avant-bras et de la pulpe du majeur. Electrodes passives au niveau du poignet et du tiers moyen de l'avant-bras.

1.3.3 L'imagerie laser Doppler (Laser Doppler Imaging, LDI) :

Il s'agit d'une méthode d'exploration du flux sanguin cutanée également non invasive mais dont l'enregistrement n'est pas continu. La technique **d'imagerie laser Doppler (LDI)**, (*figure 13*) a été conçue pour améliorer la résolution temporelle. Elle consiste à guider la lumière par un système de miroir pivotant, les photons rétrodiffusés étant amenés à des détecteurs via le même système de miroir placé près du laser. Cette technique facilite la focalisation et le suivi du spot laser lorsque le faisceau bouge, supprimant l'effet adverse de la lumière ambiante. Cependant, la durée nécessaire au balayage de la surface étudiée par le faisceau ne permet pas d'étudier des phénomènes de cinétique rapide (*figure 14*). Bien que l'hyperhémie postocclusive ait tout récemment été étudiée par cette technique, ceci n'a été possible qu'en utilisant un champ très réduit de 8 pixels par 8 pixels, soit une perte énorme de résolution spatiale (Keymel *et al.*, 2010).



Figure 13 : iontophorèse évaluée avec le LDI (tête de la camera du LDI en haut, boîtiers de iontophorèse en bas, électrodes passives au poignet et sonde de iontophorèse à proximité du faisceau laser au centre du champ d'exploration du LDI)

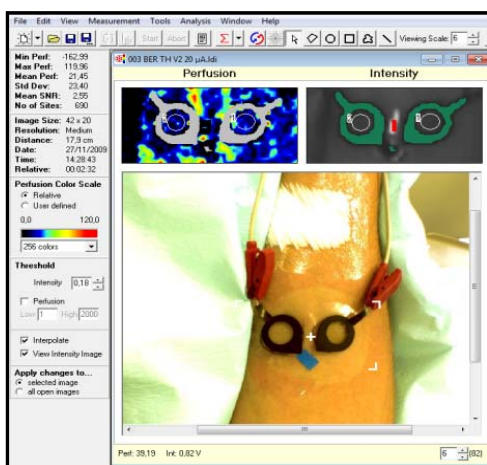


Figure 14 : Données d'acquisition en imagerie laser Doppler (collection personnelle du service)

1.3.4 L'imagerie laser de granularité (Laser Speckle Contrast Imaging, LSCI)

Plus récemment, l'imagerie laser a été utilisée en analysant la granularité du faisceau laser avec le **Laser Speckle Contrast Imaging** (LSCI). Il s'agit également d'une technique non invasive utilisée pour évaluer l'activité microcirculatoire cutanée basale et après des tests de provocation (iontophorèse, occlusion artérielle, chauffage ou refroidissement de la peau).

Basée sur le principe du speckle, elle permet de réaliser des cartographies en 2 dimensions de l'état de perfusion des tissus cutanés (*Figure 15*) avec une cinétique supérieure à celle du laser Doppler (jusqu'à 25 images.s^{-1}). La fréquence d'échantillonnage peut être variable et le plus souvent fixée à 8 Hz.

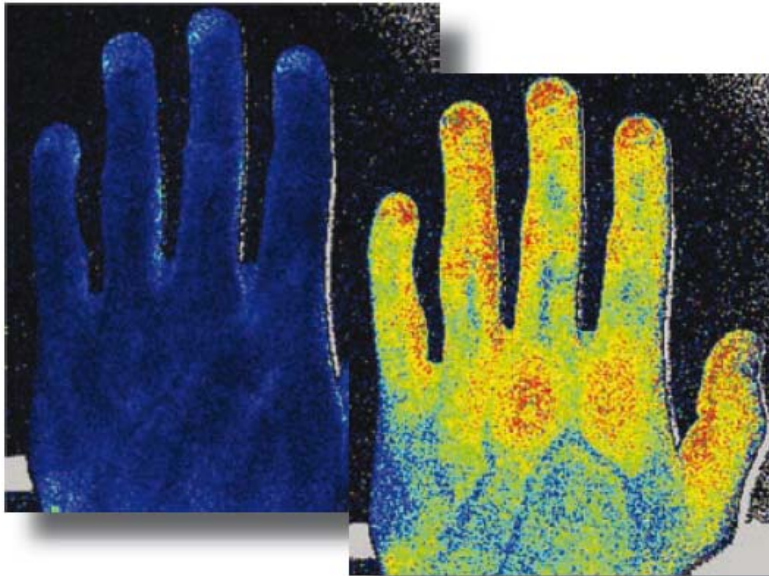


Figure 15:
Enregistrement du flux sanguin cutané en imagerie laser speckle, représenté par une échelle colorimétrique allant du bleu (flux bas, à gauche) au rouge (flux élevé, à droite). Images obtenues avec le laser speckle acquis par le laboratoire (PeriCam System, Perimed, Jarfalla, Suède ; marquage CE MDD 93/42/EEC).

Ce système est constitué d'une source laser et d'une camera digitale de haute résolution qui permet d'orienter les images. La tête du scanner peut être positionnée et immobilisée grâce à un bras amovible à une distance de 15 à 20 cm de la zone à analyser. L'échantillon est illuminé par la lumière laser pendant l'acquisition d'image à très grande vitesse. Chaque image contient un réseau de granularité légèrement différent, du fait des changements de position des éléments mobiles dans la zone d'intérêt. La variation d'intensité de chaque granularité à la même position dans chaque image permet de calculer la vitesse des éléments responsables de cette variation. Cette méthode permet donc de combiner une haute résolution temporelle à une haute résolution spatiale (*figure 16*).

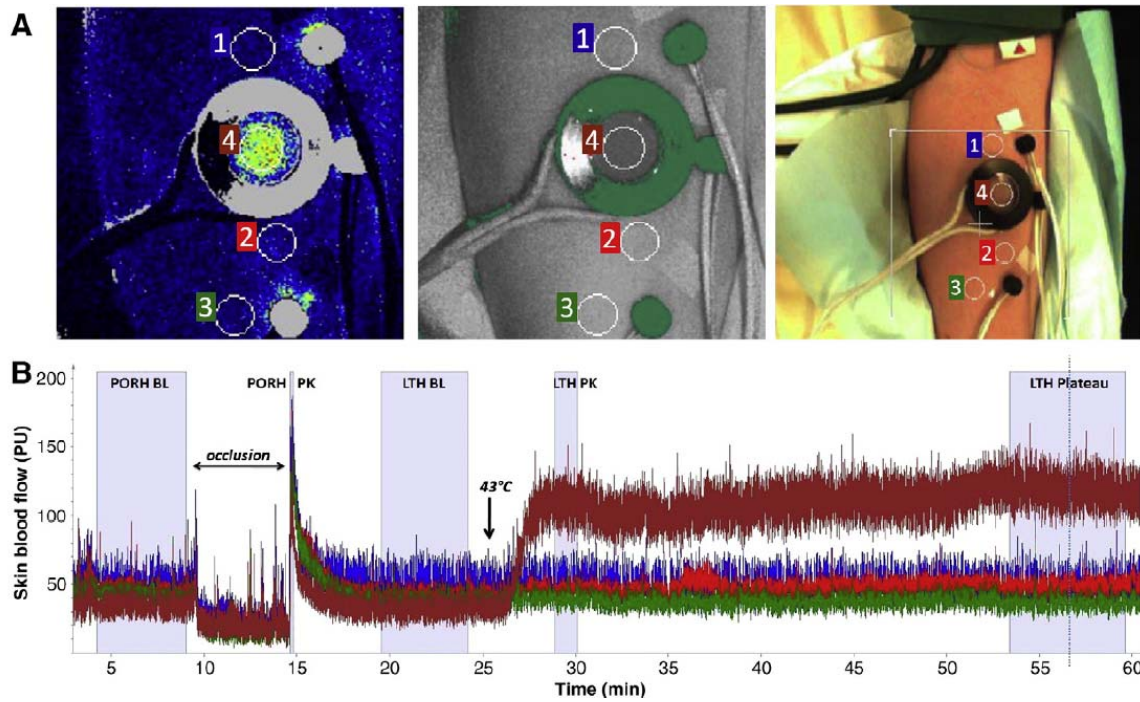


Figure 16.

(A) Perfusion cutanée évaluée en Laser speckle contrast imaging (LSCI) (gauche; échelle de flux allant de flux bas (noir) à élevé (rouge), intensité (image médiane) et photo (image de droite) sur l'avant-bras. Des régions d'intérêt (Regions of interests, ROIs) sont insérées sur la peau permettant de moyenniser un signal temporel illustré sur la partie B de l'image montrant l'effet d'une hyperhémie postocclusive, puis d'une hyperhémie thermique (Roustit et al., 2010b).

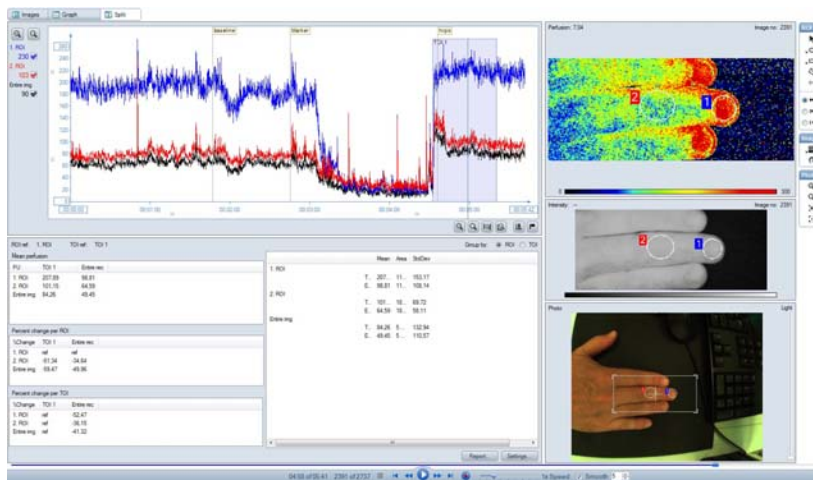


Figure 17: Acquisition du flux sanguin cutané de manière non invasive avec le LSCI lors d'une PORH. LSCI: Laser speckle contrast imaging PORH: Hyperhémie postocclusive

1.4 Les tests d'évaluations microcirculatoires : HPO ; hyperhémie post chauffage

La **dilatation flux-médiée de l'artère brachiale** (FMD) est utilisée comme un marqueur de dilatation NO-dépendante de la conductance artérielle, mais elle ne permet pas de distinguer les réponses vasculaires endothélium ou non endothélium dépendant. Différents tests sont utilisés en physiologie comme moyens d'exploration de la fonction endothéliale de la microcirculation cutanée. Les principaux tests utilisés pour les fonctions endothéliales sont **l'hyperhémie post occlusive** (post occlusive reactive hyperemia, PORH), **l'hyperhémie post chauffage local** (local thermal hyperemia, LTH) et **la iontophorèse à l'acétylcholine**. La **iontophorèse au SNP** est un test évaluant la fonction non endothéliale dépendante (Cracowski *et al.*, 2006).

1.4.1 L'hyperhémie post chauffage local (LTH)

La LTH est un moyen intéressant d'investiguer la fonction microcirculatoire (Cracowski *et al.*). Une hyperhémie en réponse à un chauffage local cutané est caractérisée par un pic initial, suivi par un nadir puis un plateau tardif. Le pic initial est presque exclusivement médié par le CGRP (calcitonine gene-related peptide) et/ou substance P, dépendant du réflexe axonal au niveau de l'avant-bras. Ce pic est émoussé par un pré traitement par de la lidocaïne/prilocaine. Par contraste, le plateau tardif est considéré comme NO-dépendant (oxyde nitrique). Ainsi la réponse cutanée microcirculatoire au chauffage local provient d'une réponse intégrant une réponse neurovasculaire et une régulation du flux cutané NO dépendant (*Figure 18*). Chez le sujet sain, la réponse du pic initial apparaît en environ 3 minutes après le début du chauffage alors que la phase plateau est atteinte en 20 à 40 minutes.

Les tests de chauffage ne sont pas toujours standardisés. Les premiers protocoles utilisés par l'équipe utilisaient une température de 42°C pendant 40 minutes puis 5 minutes à 44°C. Ce schéma méthodologique a été utilisé dans les premières études comme INFLUX IT-VS. L'expérience de l'équipe a fait modifier ce protocole pour un schéma de chauffage à 43°C, plus réaliste pour la réalisation ultérieure des protocoles chez les patients sclérodermiques (expérience d'une brûlure bénigne lors d'un chauffage chez un patient sclérodermique à 44°C). La plupart des auteurs sont d'accord pour considérer un chauffage à 43°C comme suffisant pour une dilatation maximale (Minson).

Les données concernant la LTH chez les patients avec un Raynaud secondaire montrent que la LTH est perturbée (Boignard *et al.*, 2005) en termes d'amplitude et de cinétique. Cette perturbation est présente au niveau du doigt mais pas au niveau de l'avant-bras, chez les patients ScS avec et sans fibrose cutanée et sans lien avec la présence d'une éventuelle macroangiopathie (Salvat-Melis *et al.*, 2006). La LTH avec et sans lidocaïne/prilocaine a permis d'évaluer plus précisément les anomalies de réponse chez les patients ScS orientant vers une anomalie digitale neurovasculaire (Roustit *et al.*, 2008b) (*figure 19*).

Figure 18: Tracés de LTH chez le volontaire sain avec (courbes inférieures bleue et rouge) et sans (courbes supérieures bleue et rouge) lidocaïne/prilocaine au niveau de la pulpe du doigt (en bleu) et de l'avant-bras (en rouge) montrant l'atténuation du pic initial mais pas de la phase plateau.

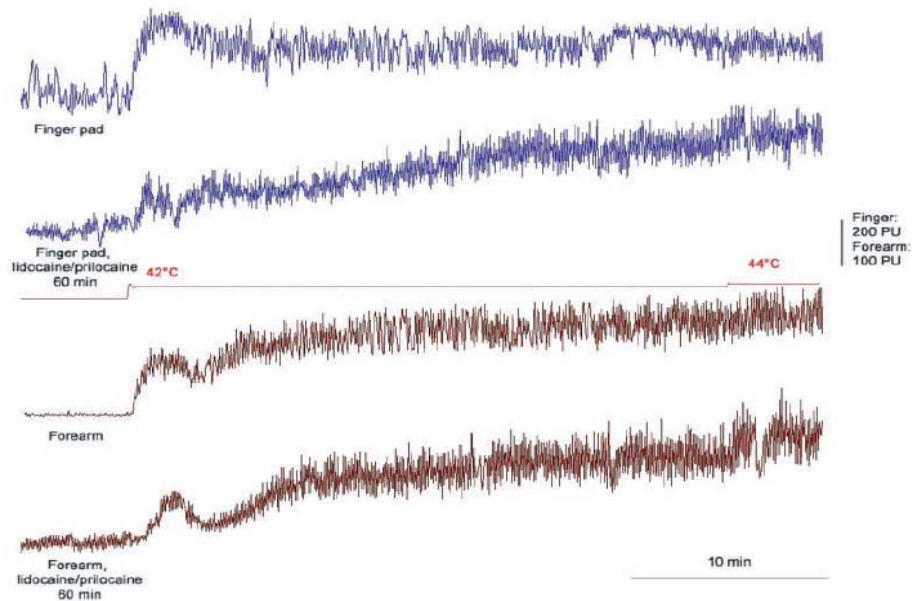
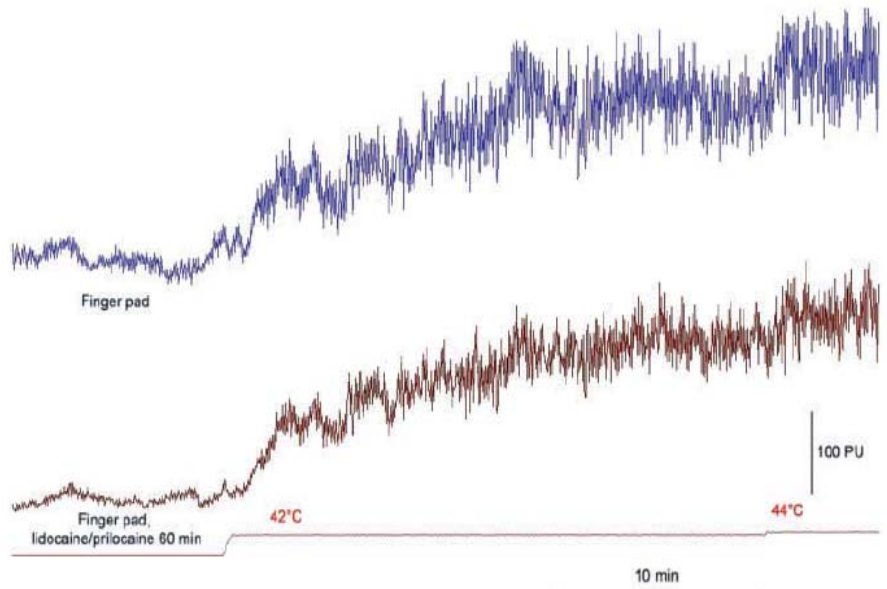


Figure 19: Tracés de LTH identiques pathologiques avec altération du pic initial chez un patient ScS avec (courbe bleue) et sans (courbe rouge) application de lidocaïne/prilocaine qui n'a pas d'effet sur le tracé. (Roustit et al., 2008b)



1.4.2 L'hyperhémie post occlusive (PORH)

L'implication du NO dans la PORH est plus controversée. Il existerait en effet une implication des facteurs hyperpolarisants dérivés de l'endothélium (EDHF). Les nerfs sensitifs et les canaux calciques de large conductance activés par le potassium joueraient un rôle majeur comme composants des EDHF dans la PORH et apparaissent comme des facteurs indépendants les uns des autres (Lorenzo *et al.*, 2007). Les résultats sont discordants en ce qui concerne l'implication de la voie des cyclooxygénases. Il existerait des interactions entre les stimulations sympathiques α_1 et α_2 et le NO lors de la PORH (Cankar *et al.*, 2009). Il a été clairement démontré que la stimulation sympathique diminuait la vasodilatation NO-dépendante au travers de la conductance des vaisseaux cutanés via la stimulation des adrénorécepteurs α_1 . Au niveau de la microcirculation de l'avant-bras chez l'homme, le NO endogène inhibe la vasoconstriction via le refroidissement de l'ensemble du corps et la norépinéphrine.

Le principal écueil pour utiliser ces explorations dans des études cliniques plus larges est le manque de standardisation de l'expression du flux cutané sanguin, notamment en ce qui concerne le flux de base (baseline) de référence (*Figure 20*).

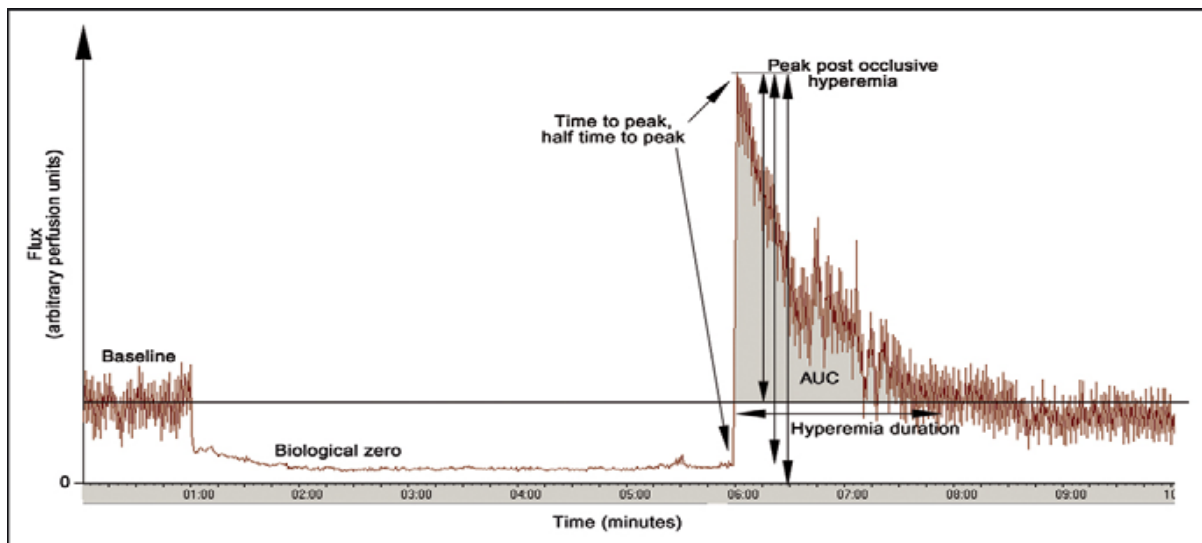


Figure 20: tracé typique de réponse à une HPO où les principaux paramètres sont enregistrés. Le zéro biologique correspond à l'enregistrement du signal doppler en absence de flux sanguin. Un seul paramètre comme celui de l'amplitude du pic de l'hyperhémie est exprimée en valeur absolue valeur absolue moins le zéro biologique, valeur absolue moins la baseline, ou valeur absolue divisée par la baseline. Elle peut également être exprimée en pourcentage de vasodilatation maximale induite par un chauffage local à 42 ou 44 °C. (Cracowski et al., 2006). AUC : area under the curve

L'étude de Roustit a permis d'étudier la corrélation entre les atteintes macrovasculaires et microvasculaires dans trois groupes de patients (33 volontaires sains, 36 Raynaud primaire et 42 ScS) en étudiant simultanément l'HPO, la FMD (dilatation flux médiée de l'artère brachiale) et la LTH (Roustit *et al.*, 2008a). La PORH était altérée chez les RP primaires et les ScS et la LTH uniquement chez les ScS. Il existait une perte de corrélation entre la FMD et la PORH chez les patients RP primaires et ScS.

1.4.3 la iontophorèse comme test physiologique

1.4.3.1 Iontophorèse d'acétylcholine

La iontophorèse d'acétylcholine est couramment considérée comme un test de la fonction endothéliale. Il existe cependant une incertitude concernant les médiateurs de la vasorelaxation induite, le NO ne semblant pas impliqué, contrairement aux COX.

1.4.3.2 Iontophorèse de SNP

La iontophorèse de SNP est utilisée comme un test physiologique pour évaluer la fonction microvasculaire cutanée non endothélium dépendante et est réalisée le plus souvent au niveau de l'avant-bras. Le SNP est un donneur de NO. La vasodilatation induite par le SNP n'est pas liée à la production endothéliale de NO mais agit comme « prodrogue » donneur de NO,

induisant l'activation de la guanylate cyclase et l'augmentation des concentrations cellulaires de cGMP dans la cellule musculaire lisse.

La iontophorèse de SNP a été réalisée chez des patients ScS (comparés à des volontaires sains) comme technique pour évaluer la fonction microvasculaire cutanée non endothélium dépendante (Roustit *et al.*, 2009). La iontophorèse de SNP induit une hyperhémie au niveau de l'avant-bras qui n'est pas affectée par le pré traitement par la lidocaïne /prilocaine (réponse non axonale réflexe dépendante), par contre, il n'a pas été observé d'augmentation de la conductance cutanée vasculaire au niveau de la pulpe du doigt. Cette méthode n'est donc pas applicable pour l'exploration de la pulpe du doigt chez le ScS.

1.4.4 La microdialyse

La microdialyse intra dermique est la méthode la plus appropriée pour administrer des outils pharmacologiques pour étudier la microcirculation. Le principal biais est que les fibres de microdialyse ne peuvent être administrées dans tous les territoires cutanés du fait de la iatrogénicité, et notamment au niveau des doigts (*Figure 21*). Elle apparaît donc plus comme un outil d'exploration (test physiologique ou analyse pharmacocinétique) mais pas comme un futur outil thérapeutique, notamment dans la sclérodermie où il faut éviter la iatrogénicité et notamment au niveau des doigts. Certaines équipes ont développé une technique de microinjection intradermique avec analyse par fluxmétrie laser doppler (Cankar *et al.*, 2009). Cette technique, même si elle rend certains territoires explorables plus accessibles, reste encore invasive et n'est utilisée que dans le cadre d'études de compréhension de réactions physiologiques nécessitant un blocage pharmacologique.

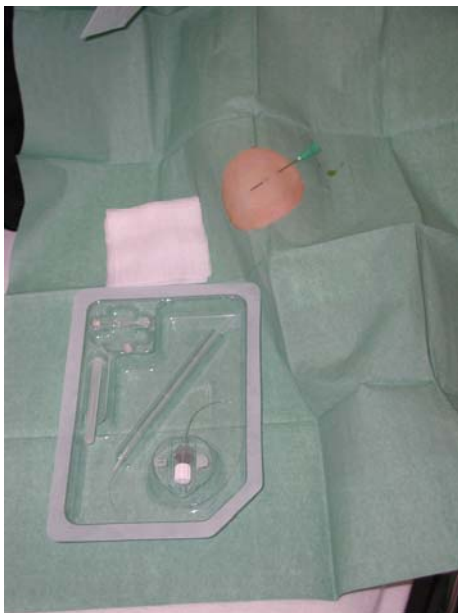


Figure 21: technique de microdialyse avec cathétérisme intra dermique au niveau de l'avant-bras (iconographie du service du Centre d'Investigation Clinique CHU Grenoble)

1.5 Base pharmacologique des molécules testées

Les molécules qui ont été sélectionnées pour développer la iontophorèse comme axe thérapeutique dans la ScS se veulent d'avoir comme point commun des propriétés vasodilatatrices et d'avoir été testées dans l'HTAP (*figure 22*). Comme exposé précédemment, le passage d'une molécule en iontophorèse est conditionné par de nombreux paramètres, notamment les propriétés physico-chimiques des molécules. Certains éléments

vont être présentés expliquant le choix stratégique des molécules sélectionnées pour nos protocoles en iontophorèse.

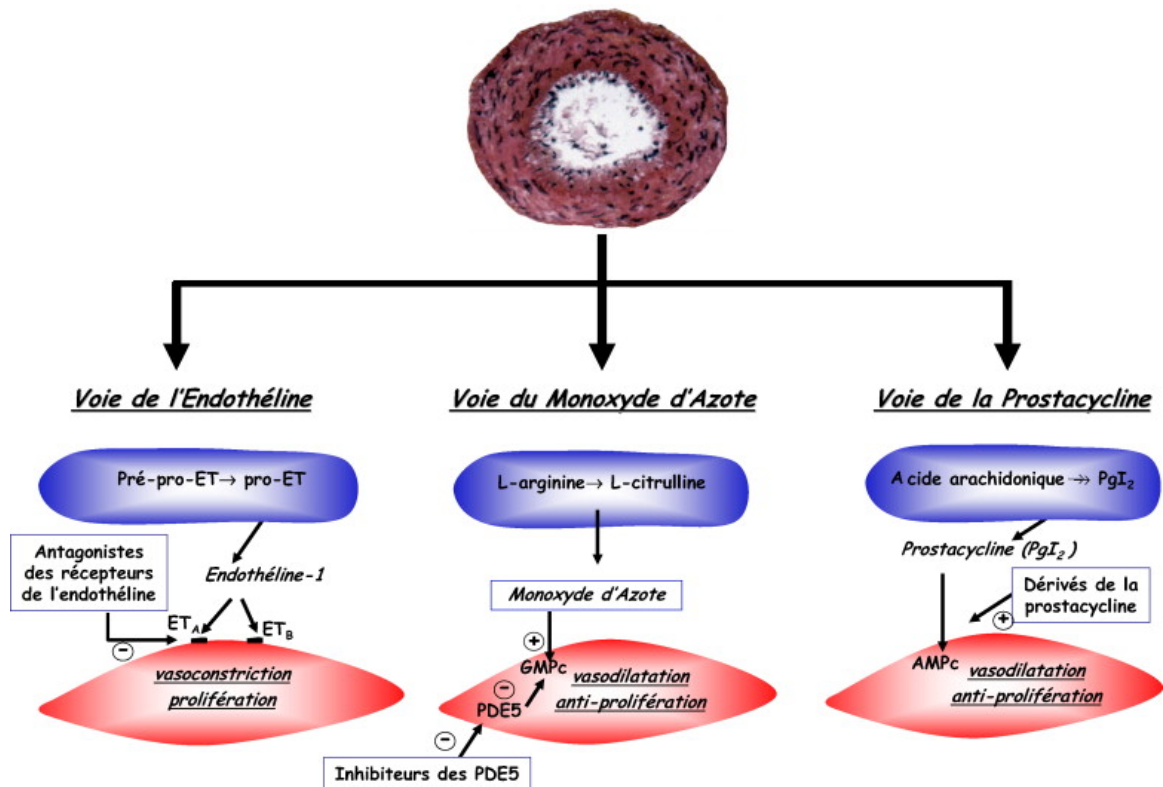


Figure 22 : Cibles des traitements disponibles et en voie de développement dans l'hypertension artérielle pulmonaire

1.5.1 La voie du monoxyde d'azote

La trinitrine ou nitroglycérine a été utilisée comme traitement local en galénique pommade ou en patch transdermique dans le traitement du RP. Ce traitement a été arrêté du fait des effets secondaires notamment des malaises et des maux de tête liés à l'effet systémique. L'administration de nitroglycérine en topique permet néanmoins de diminuer la sévérité et la fréquence des épisodes de RP primaire ou secondaire (Levien). Une récente formulation topique de nitroglycérine est en évaluation dans différentes études cliniques (soumission en cours à la FDA). Il s'agit de MQX-503 0.5% et 1.25 % (MediQuest Therapeutics, Inc., Bothell, Washington, USA), une microémulsion de nitroglycérine avec approximativement 50% de phase organique à base de lécithine et 50% de phase aqueuse. L'absorption est très rapide. Elle permet selon les études de réduire la sévérité des crises de RP primaire mais pas la fréquence ou la durée (Levien). On sait néanmoins la difficulté à mettre en place des études de bonne méthodologie dans l'évaluation des traitements dans le RP. Une étude a été réalisée dans le RP secondaire chez les patients ScS avec MQX-503 0.9% (versus placebo) en appliquant la pommade avant ou 5 minutes maximum après le début de la crise du RP. Le nombre moyen et la durée des RP n'étaient pas différents entre les traitements. Les ulcérations digitales (UD) apparaissaient de manière identique entre les deux groupes (Chung *et al.*, 2009). La voie du NO est néanmoins intéressante à développer via d'autres molécules impliquées dans la cascade de signalisation et/ou biotransformation du NO.

1.5.1.1 Nitroprussiate de sodium

Le nitroprussiate de sodium (Sodium nitroprussiate, SNP) est un donneur de NO. La vasodilatation induite par le SNP n'est donc pas liée à la production endothéliale de NO mais à une production liée à l'activation directe de la guanylate cyclase soluble et de l'augmentation du cGMP dans la cellule musculaire lisse (*Figure 22*).

Le SNP est un vasodilatateur indiqué dans l'hypertension artérielle (HTA) (notamment l'HTA maligne) mais également dans l'insuffisance cardiaque aiguë en particulier ventriculaire gauche avec bas débit cardiaque et résistances périphériques élevées réfractaires aux traitements habituels (infarctus myocardique, cardiomyopathies, dysfonctionnement valvulaire aortique et mitrale et au cours de la chirurgie coronarienne et valvulaire).

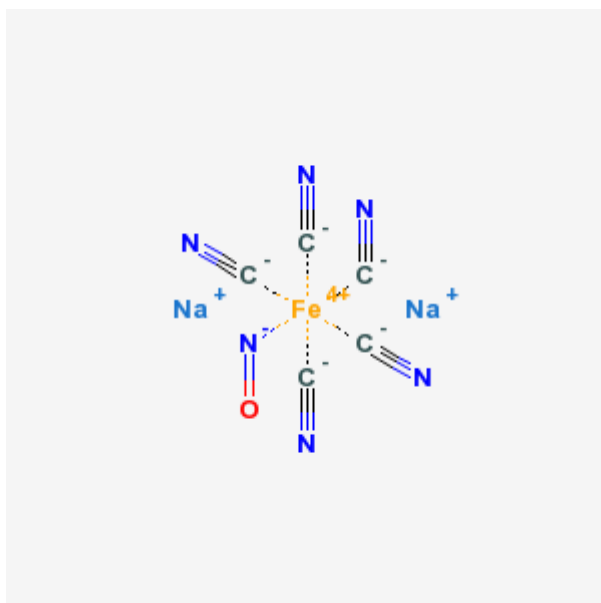


Figure 22 : structure chimique du SNP

Le SNP est utilisé en iontophorèse comme test pharmacologique. Il a également été testé chez les patients ScS où il a été démontré qu'il induisait une augmentation de la conductance vasculaire cutanée (Anderson, Moore et al. Rheumatology 2004). La iontophorèse de SNP a donc été proposée comme traitement des UD mais ne nous semble pas être un bon candidat du fait de sa demi-vie très courte. Sa demi-vie d'élimination étant très brève (1 à 3 minutes), le schéma thérapeutique proposé devrait être un traitement continu, peu pratique. L'effet du SNP sur les vaisseaux peut également dépendre de la taille des vaisseaux, avec un effet minimal pour des vaisseaux inférieurs à 100µm et maximal supérieur à 200µm (The Pharmacological Basis of Therapeutics, Goodman et Gilman's, 11th edition, Mc Graw Hill). Dans la plupart des études proposées ici, la iontophorèse de SNP est donc utilisée comme un « témoin positif » des expériences, même si l'objectif est de trouver une molécule diffusée en iontophorèse avec un meilleur résultat en terme d'efficacité ou de meilleure pharmacocinétique sur la vasodilatation cutanée.

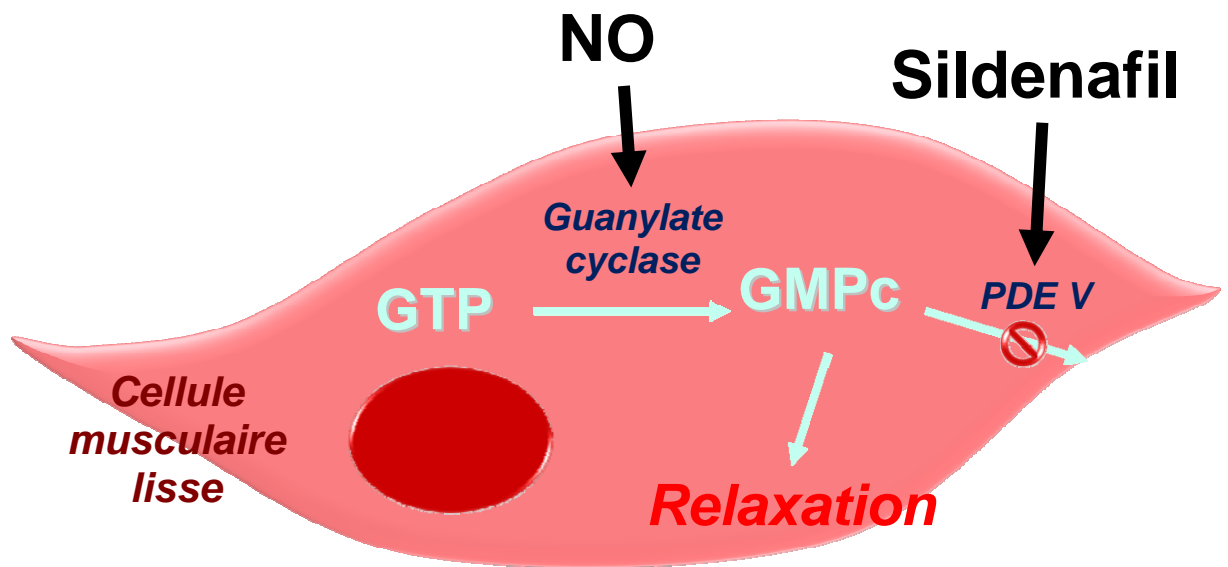
1.5.1.2 Sildénafil

Le NO vasodilate et inhibe l'activation plaquettaire via la guanosine cyclique 5'-monophosphate (cGMP). La cGMP est hydrolysée par les phosphodiesterases, en particulier

la phosphodiesterase-5, isoenzyme spécifique de la cGMP. Le Sildenafil, le tadalafil et le vardenafil sont des inhibiteurs spécifiques de la phosphodiesterase de type 5 ayant pour conséquence l'augmentation de cGMP et la dilatation micro et macrovasculaire (*Figure 23*)

Le Sildénafil (commercialisé sous le nom de Viagra® ou Revatio®) est un inhibiteur puissant et sélectif de la phosphodiesterase de type 5A (PDE5A). Outre la présence de cette enzyme dans les corps caverneux du pénis, la PDE5 est également présente dans le système vasculaire pulmonaire. Par conséquent, le sildénafil augmente les concentrations de GMPc présente au sein des cellules musculaires lisses des vaisseaux pulmonaires, ce qui se traduit par une relaxation du muscle lisse. Chez les patients présentant une hypertension artérielle pulmonaire, cet effet peut conduire à une vasodilatation du lit vasculaire pulmonaire et, dans une moindre mesure, à une vasodilatation de la circulation générale. Son effet est plus puissant sur la PDE5 que sur les autres phosphodiésterases connues (sélectivité 10 fois plus importante par rapport à la PDE6, impliquée dans le processus de phototransduction de la rétine. sélectivité 80 fois par rapport à la PDE1 et de plus de 700 fois par rapport aux PDE2, 3, 4, 7, 8, 9, 10 et 11). En particulier, le sildénafil est plus de 4000 fois plus sélectif pour la PDE5 que pour la PDE3, l'isoforme de la phosphodiésterase spécifique de l'AMPc impliquée dans le contrôle de la contractilité cardiaque. Le sildénafil entraîne des diminutions faibles et transitoires de la pression artérielle, dans la plupart des cas sans retentissement clinique.

Figure 23: Mode d'action schématique du sildénafil au niveau de la cellule musculaire lisse



Les inhibiteurs de PDE5A ont un effet vasodilatateur pulmonaire démontré lors de tests de vasodilatation effectués chez des patients présentant une hypertension artérielle pulmonaire (HTAP). L'autorisation de mise sur le marché du sildénafil est l'HTAP chez les patients en classe fonctionnelle II et III selon la classification de l'OMS. Une étude randomisée en double insu contre placebo incluant 178 patients atteints d'HTAP a montré l'efficacité du sildénafil sur la tolérance à l'exercice et l'hémodynamique (Galie *et al.*, 2005). Ce bénéfice sur le test de marche de six minutes était maintenu à un an de traitement. Le sildénafil a l'intérêt de son

administration par voie orale (trois prises par jour) et de sa bonne tolérance clinique. Des diarrhées, des dyspepsies et des flushs peuvent survenir.

Le sildénafil a ensuite été testé dans les Raynaud sévères en 2ème ligne (Levien, 2006) et dans les ulcérations digitales en étude ouverte (Colglazier et al., 2005) et lors d'un essai clinique randomisé en double aveugle (Fries et al., 2005). Cette dernière étude en double aveugle versus placebo en cross over testait le sildenafil dans les RP résistants aux traitements vasodilatateurs (inhibiteurs calciques chez 94% des patients, nitroglycerin dans 39%, pentoxifylline dans 28%, inhibiteur de l'enzyme de conversion dans 22%, prostaglandines intraveineuses dans 17%, et bosentan dans 6%); 16 patients étaient inclus avec RP secondaires et 2 patients avec RP primaire. On observait une réduction en fréquence et en durée des crises de RP sous sildénafil et 6 patients avec UD cicatrisées (2 cas de récurrence sous traitement).

Une étude prospective monocentrique avec 19 patients ScS et 49 ulcérations digitales confirme l'effet du sildenafil per os qui était utilisé de 60 mg /jour jusqu'à 150 mg/jour jusqu'à 6 mois avec 17 ulcérations digitales à la fin de l'étude (à noter l'apparition de 12 UD sous traitement par sildenafil) (Brueckner *et al.*). Des résultats discordants ont néanmoins été rapportés avec aucun bénéfice chez 20 patients avec RP primaires traités par sildenafil 50 mg 2 fois par jour pendant 2 semaines versus placebo dans une étude randomisée en double aveugle et en cross over (Zamiri B et al.)

En ce qui concerne les autres molécules inhibitrices PDE5, une étude ouverte non randomisée a testé le tadalafil chez 12 patients avec RP secondaire et UD douloureux ou nécrose digitale avec un temps moyen de cicatrisation des UD de 5 semaines et de nécrose digitale de 8 semaines. Une étude ouverte randomisée en double aveugle en crossover incluait 25 patients avec RP secondaire avec une fréquence et une durée et le Raynaud's Condition Score (RCS) score améliorés dans le groupe tadalafil (Shenoy *et al.*). Comme avec le sildénafil, les résultats sont discordants avec une étude randomisée en double aveugle concluant à l'inefficacité du tadalafil avec 29 patients avec RP secondaire (Schiopu *et al.*, 2009).

1.5.2 Voie des antagonistes de l'endothéline

Des études assez récentes ont montré que les antagonistes des récepteurs aux endothélines pouvaient être une alternative de traitement aux prostaglandines chez les patients avec des RP sévères résistants aux autres traitements. L'activation des récepteurs de l'endothéline A (ET-A) et B (ET-B) situés au niveau des cellules musculaires lisses de la paroi des artères pulmonaires aboutit à une vasoconstriction, une prolifération cellulaire et à une hypertrophie. Les récepteurs ETA sont situés principalement dans les cellules musculaires lisses vasculaires et induisent une vasoconstriction et une prolifération cellulaire. Les récepteurs endothéliaux ET-B de l'endothélium peuvent, quant à eux, lorsqu'ils sont activés, entraîner une majoration de la synthèse et de la libération de substances vasodilatatrices tels que le monoxyde d'azote et la prostacycline. Les récepteurs ETB sont situés sur les cellules endothéliales.

Ces médiateurs, en plus de leur effet vasodilatateur pulmonaire, peuvent inhiber la prolifération des cellules endothéliales et des cellules musculaires lisses vasculaires pulmonaires.

1.5.2.1 Le Bosentan

Le bosentan (Tracleer®) est un antagoniste mixte non sélectif des récepteurs de l'endothéline A (ETA) et de l'endothéline B (ETB), actif par voie orale. Il réduit la vasoconstriction médiée par l'endothéline endogène. Le bosentan a l'AMM dans l'HTAP idiopathique où une étude a

montré une amélioration de la survie à 12 et 24 mois chez les patients atteints d'HTAP idiopathiques traités en première intention par bosentan (McLaughlin *et al.*, 2005). Il a également l'AMM dans la prévention de la récurrence des UD chez les patients ScS. Une étude randomisée en double aveugle versus placebo avec 122 patients ScS a permis de mettre en évidence une réduction de 48% des nouveaux UD sans effet sur la cicatrisation. (Korn *et al.*, 2004) (Matucci-Cerinic *et al.*). Le traitement par bosentan est contre-indiqué en cas d'insuffisance hépatique modérée ou sévère ou en cas d'élévation des transaminases à plus de trois fois la normale. Le passage via la iontophorèse pourrait permettre potentiellement de limiter cet effet secondaire.

1.5.2.2 Sitaxentan

Le sitaxentan est un inhibiteur sélectif du récepteur ETA. Bien que le sitaxentan interagisse avec le métabolisme des antivitaminés K, via le cytochrome P450, exposant ainsi les patients à un risque de surdosage, peu d'incidents de ce type avaient été signalés lors des études du sitaxentan dans l'HTAP. Le sitaxentan avait donc une AMM dans l'HTAP mais la molécule a été retirée du marché en décembre 2010 du fait d'hépatotoxicités létales.

1.5.3 Les Prostanoides

La famille des prostanoides contient plusieurs molécules dont la thromboxane A₂, les prostaglandines et la prostacycline (PGI₂). La thromboxane A₂ est un puissant vasoconstricteur dans tous les lits circulatoires. De manière générale, on peut considérer que les prostaglandines sont vasodilatatrices et induisent donc une diminution de la pression artérielle. Cette dilatation concerne les artérioles, les sphincters pré-capillaires et les veinules post-capillaires. Certaines prostaglandines sont vasoconstrictrices. En fait, l'effet cardiovasculaire d'une prostaglandine dépend d'au moins trois éléments : sa nature, la dose utilisée et le lit circulatoire considéré. La PGD₂ est vasodilatatrice à doses faibles dans tous les lits vasculaires, y compris les lits mésentérique, coronaire et rénal. A dose plus forte, elle devient vasoconstrictrice. Quant au lit circulatoire pulmonaire, il constitue une exception puisque la PGD₂ y est toujours vasoconstrictrice. La PGF₂ α induit des augmentations de la pression artérielle dans de nombreux modèles expérimentaux. Chez l'homme, elle est veinoconstrictrice et augmente la pression artérielle pulmonaire. En revanche, elle augmente peu la pression artérielle systémique. Les prostaglandines des séries E et F sont vasodilatatrices, tachycardisantes et inotropes positives. Ces deux derniers effets sont largement dus à une activation baroréflexe consécutive à la réduction des résistances vasculaires périphériques.

L'injection intraveineuse de PGI₂ induit une hypotension majeure qui est environ 5 fois plus importante que celle induite par la PGE₂. Cette hypotension est accompagnée d'une tachycardie réflexe. La prostacycline est produite par les cellules de l'endothélium vasculaire et contribue à l'effet antithrombotique de l'endothélium intact par inhibition de l'agrégation plaquettaire. Elle est produite par l'action de l'enzyme prostacycline synthétase sur la prostaglandine H₂. La prostacycline est appelée PGI₂ (prostaglandine I₂) dans la nomenclature des eicosanoïdes, et est un membre des prostanoides (avec les prostaglandines et le thromboxane). La prostaglandine PGH₃ peut elle aussi subir l'action de la prostacycline synthétase et conduit alors à une autre prostacycline PGI₃. Le terme 'prostacycline' réfère habituellement à la PGI₂. La PGI₂ est issue de l'acide arachidonique (oméga-6). La PGI₃ est issue de l'acide eicosapentaénoïque (oméga-3). Les PGI₂ ont une action relaxante sur les muscles lisses. Il existe des familles de récepteurs pour chaque groupe de prostanoides

(prostaglandines, leukotriènes). La grande diversité des effets biologiques découle en grande partie de la grande hétérogénéité des récepteurs qui constituent cette famille de récepteurs à 7 domaines transmembranaires couplés à des protéines G. Chaque récepteur est dénommé en fonction de la prostaglandine qui présente le plus d'affinité pour lui. Ainsi, il existe 5 groupes de récepteurs : DP (pour les PGDs), FP (pour les PGFs), IP (pour la PGI₂), TP (pour le TXA₂) et EP (pour les PGEs). Pour la PGI₂, le second messenger induit une augmentation de l'AMP cyclique. L'augmentation de la production d'AMPc intracellulaire relâche le muscle lisse vasculaire et inhibe l'agrégation plaquettaire.

La première publication de l'efficacité d'une prostacycline dans l'HTAP date de 1982, confirmée en 1996 par une efficacité supérieure au traitement conventionnel. Le challenge dans le domaine de la recherche est de proposer des modifications chimiques qui augmenteraient la stabilité de la molécule sans perte des propriétés pharmacologiques, d'où le développement de molécules analogues de synthèse telles que l'iloprost mais également le tréprostinil, le beraprost, le ciprostone et le cicaprost. Les analogues de la prostacycline exercent leurs effets en activant le récepteur IP. Mais la majorité des analogues de PGI₂ peuvent activer d'autres récepteurs prostanoïdes. Actuellement, tous les agonistes IP commercialisés sont de structure prostanoïde. En parallèle, des agonistes IP présentant une autre structure chimique sont en cours de développement, principalement pour permettre une résorption digestive.

1.5.3.1 La prostacycline

La PGI₂ est un puissant vasodilatateur systémique et artériel pulmonaire ainsi qu'un inhibiteur de l'agrégation plaquettaire. Le terme de prostacycline est généralement réservé à la substance endogène. La prostacycline synthétique, de structure chimique identique, utilisée comme médicament, est l'époprostenol (Flolan[®]). La molécule est instable au pH physiologique et les solutions sont généralement préparées avec une solution tampon alcaline à pH 10.5. La demi-vie en solution aqueuse à pH 7.4 est de moins de 3 minutes à 37°.

L'époprostenol agit sur le remodelage vasculaire et améliore la tolérance à l'effort en diminuant la pente de la courbe pression-débit dans la circulation pulmonaire (Rubin *et al.*, 1990). La prostacycline intraveineuse a été utilisée comme traitement de l'HTAP pour la première fois au début des années 1980 et a depuis prouvé son efficacité sur la survie et l'amélioration fonctionnelle. Après trois mois de traitement par époprostenol, la dyspnée et la distance parcourue lors du test de marche constituent des indices pronostiques (meilleure survie pour les patients revenus en classe fonctionnelle I-II ou parcourant une distance supérieure à 380 m en six minutes) (Sitbon *et al.*, 2002). L'époprostenol est administré par voie intraveineuse continue à l'aide d'une pompe connectée à un cathéter tunnelisé placé au niveau de la veine sous-clavière. Les effets secondaires sont fréquents, en particulier les douleurs des mâchoires, les céphalées, les diarrhées, les bouffées de chaleur, les douleurs des membres inférieurs et les épisodes de nausées ou de vomissements. Les complications les plus sévères sont liées au mode d'administration, la perfusion continue par cathéter pouvant se compliquer de thromboses ou d'infections (incidence des sepsis de l'ordre de 0,1 à 0,4 cas par an et par patient) (Robbins *et al.*, 1998). L'interruption du traitement par dysfonction de la pompe ou rupture de cathéter peut se compliquer d'une aggravation brutale de l'HTAP potentiellement mortelle du fait de la très courte demi-vie du produit (environ 45 minutes).

1.5.3.2 Analogues de la prostacycline

1.5.3.2.1 *Treprostinil*

Le tréprostinil (Remodulin®) est un analogue de la prostacycline administré rapidement et complètement par voie sous-cutanée à l'aide d'un système de mini-pompe semblable à celui utilisé pour la délivrance de l'insuline chez le diabétique.

Il exerce un effet vasodilatateur direct au niveau de la circulation artérielle pulmonaire et systémique ainsi qu'un effet inhibiteur de l'agrégation plaquettaire. Chez l'animal, les effets vasodilatateurs réduisent la post-charge des ventricules droit et gauche tout en augmentant le débit cardiaque et le volume d'éjection systolique. Chez l'animal, l'effet du tréprostinil sur le rythme cardiaque dépend de la dose et il n'a pas été observé d'effet notable sur la conduction cardiaque.

En cas de mauvaise tolérance, l'administration intra veineuse peut être proposée. Le tréprostinil a des propriétés vasodilatatrices et d'inhibition de l'agrégation plaquettaire. Il est métabolisé par le foie et une demi-vie de 4 heures environ. Son poids moléculaire est de 412.49 g/mol et sa formule C₂₃H₃₃NaO₅ (Figure 24)

Le Remodulin® est la forme commerciale qui contient 1 mg/mL, 2.5 mg/mL, 5 mg/mL ou 10 mg/mL de treprostinil. Son efficacité à 12 semaines a été évaluée chez des patients atteints d'HTAP idiopathique, liée à une cardiopathie congénitale ou à une connectivite, en classe fonctionnelle II, III ou IV de la New York Heart Association (NYHA). Après 12 semaines, le tréprostinil améliorait significativement la distance parcourue en six minutes (+16 min), les scores de dyspnée, les symptômes ainsi que les paramètres hémodynamiques. Les douleurs au point d'injection observées chez 85 % des patients constituaient un facteur limitant à l'augmentation des doses et conduisaient à l'arrêt du traitement dans 8 % des cas (Simonneau *et al.*, 2002). Avec l'expérience, les praticiens ont remarqué que certains sites d'administration étaient mieux tolérés que d'autres (abdomen, flancs, face post du bras, haut des fesses ou extérieur des cuisses) mathier. (Mathier *et al.*) L'apex de la douleur survient généralement 2 à 5 jours après le début d'un nouveau site d'administration puis la douleur augmente après plusieurs semaines de traitement. Une rotation des sites d'administration améliore la tolérance. La douleur au site d'injection n'est pas corrélée à la dose administrée et une rapide titration semble avoir un effet favorable sur la douleur au site d'administration. Les effets secondaires locaux ne doivent ainsi pas limiter l'escalade de dose et l'administration sous cutanée de la molécule doit être accompagnée de traitements antalgiques locales ou systémiques (Mathier *et al.*). Le tréprostinil a donc une AMM depuis 2002 dans l'HTAP par voie sous cutanée et par inhalation en 2009. Il est indiqué dans le traitement de l'hypertension artérielle pulmonaire primitive chez les patients en classe fonctionnelle III de la NYHA. Son administration est pour l'instant sous-cutanée et intra veineuse exclusivement. Le développement de la molécule per os est en phase II d'études cliniques dans le traitement de l'HTAP (étude FREEDOM).

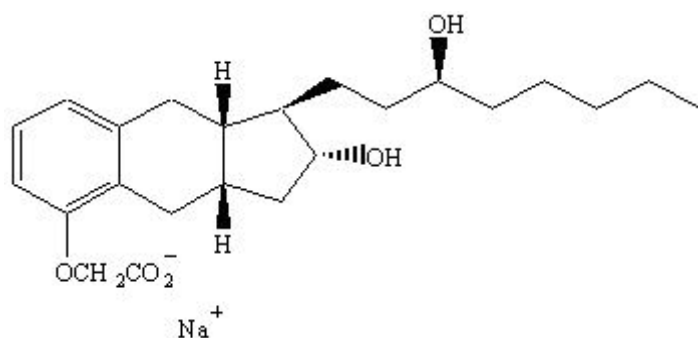


Figure 24 : structure pharmacologique du tréprostinil C₂₃H₃₄O₅

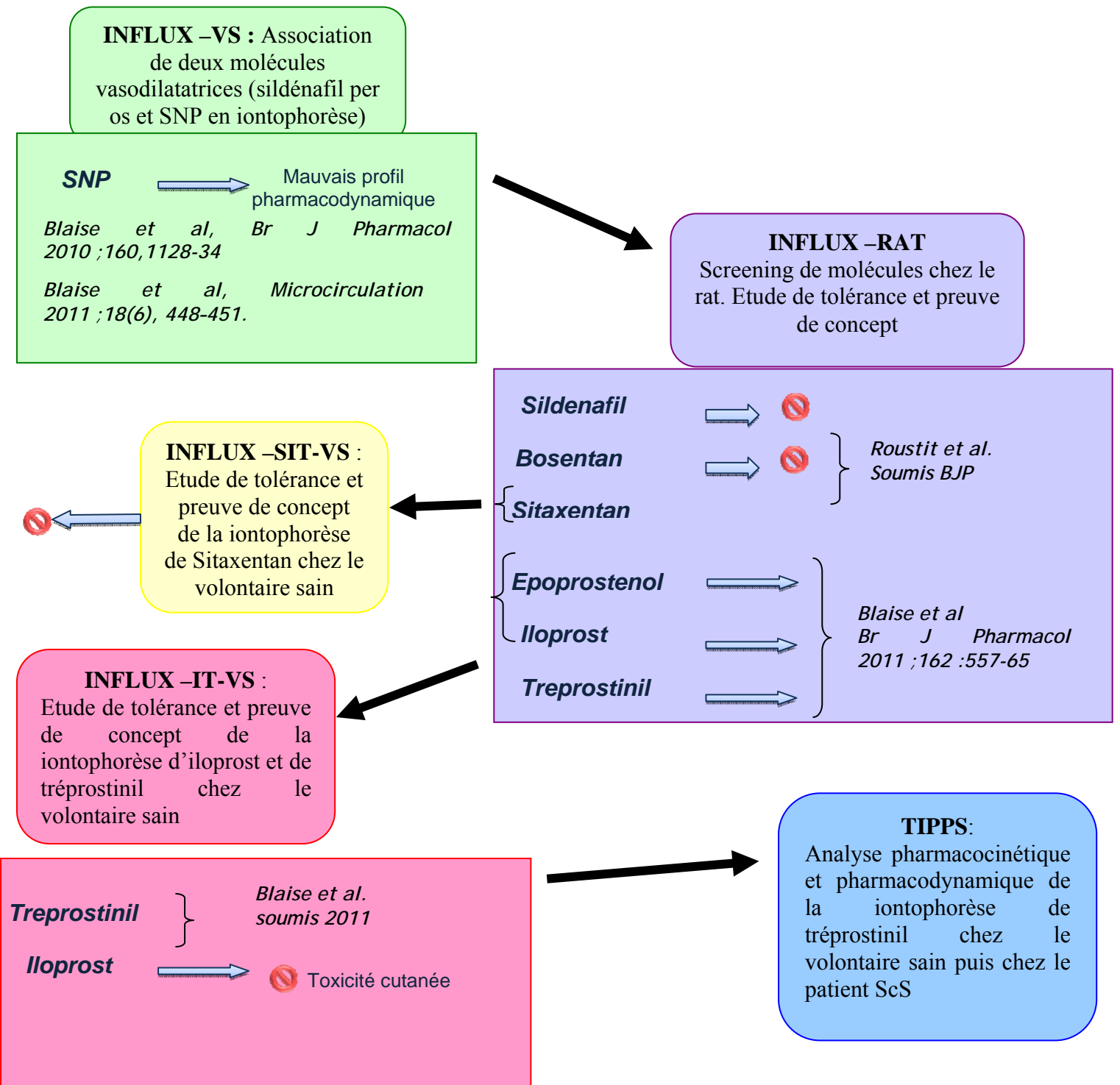
1.5.3.2.2 L'Iloprost.

L'iloprost est un analogue de la prostacycline administré par inhalation ou par voie intra veineuse. Il s'agit d'un agoniste IP. Il possède une action vasodilatatrice et anti-agrégante médiée par l'activation de récepteurs transmembranaires spécifiques. Les effets pharmacologiques suivants ont été observés : inhibition de l'agrégation plaquettaire, de l'adhésion et de la sécrétion plaquettaires, dilatation des artérioles et des veinules, augmentation de la perfusion du réseau capillaire nutritif et diminution de l'hyperperméabilité vasculaire au niveau de la microcirculation, activation de la fibrinolyse, inhibition de l'adhésion et de la migration des leucocytes après une lésion endothéliale, diminution de la libération de radicaux libres d'oxygène.

L'iloprost est indiqué dans les ischémies critiques des membres inférieurs, les RP graves avec UD et l'HTAP (forme inhalée). La courte durée d'action de l'iloprost constitue le principal désavantage de ce mode d'administration puisqu'il nécessite soit une hospitalisation pour une perfusion continue intra veineuse, soit la réalisation de 6 à 12 inhalations par jour (Hoepfer *et al.*, 2000). Une étude multicentrique randomisée a analysé le bénéfice de ce traitement après 12 semaines chez des patients atteints d'HTAP idiopathiques, liées à une connectivite ou un cœur pulmonaire chronique post embolique non opérable en classe fonctionnelle III ou IV de la NYHA. Une amélioration significative de la distance parcourue lors du test de marche (+36 min) et de la classe fonctionnelle NYHA était observée chez 17 % des sujets traités contre 4 % dans le groupe placebo. La toux et les symptômes liés à la vasodilatation représentaient les effets secondaires les plus fréquemment observés, mais un nombre de syncopes significativement plus important était rapporté dans le groupe traité (Olschewski *et al.*, 2002). Le traitement est également reconnu comme efficace dans le traitement des RP secondaires dans la ScS, diminuant la fréquence et la sévérité des crises et dans la prévention et la cicatrisation des UD (Scorza *et al.*, 2001).

Une étude en double aveugle n'a pas montré de différence entre le beraprost oral et le placebo dans le traitement du RP primaire (Vayssairat, 1996).

SCHEMA ORGANISATIONNEL DES ETUDES



2 PREMIERE PARTIE : Association d'une iontophorèse cutanée et d'un traitement vasodilatateur per os

2.1 1^{ère} étude INFLUX VS : étude chez le volontaire sain de l'association d'une iontophorèse de SNP et de l'administration per os du sildénafil à 50 ou 100 mg.

L'objectif global du projet de recherche est de développer des nouvelles voies thérapeutiques dans la ScS en développant la iontophorèse thérapeutique. La première piste était d'utiliser la technique de iontophorèse avec le SNP, molécule déjà utilisée en iontophorèse comme test physiologique et utilisée en routine par le laboratoire. Le SNP n'est néanmoins pas un bon candidat à une iontophorèse thérapeutique du fait de sa durée de vie très courte. L'idée était de potentialiser l'effet de la iontophorèse au SNP avec une molécule per os d'action synergique, le sildénafil, bien que le SNP soit une molécule vasodilatatrice contre-indiquée en association avec les vasodilatateurs donneurs directs ou indirects de NO. L'étude INFLUX-VS consiste en la réalisation chez le volontaire sain d'une iontophorèse de SNP 20 minutes à 20 μ A au niveau de l'avant-bras en association avec une prise orale de sildénafil de 50mg ou 100mg une heure auparavant, association qui n'avait jusque là jamais été testée. Les objectifs de cette étude étaient doubles : étudier la tolérance systémique de cette association et démontrer qu'une telle administration pouvait permettre une vasodilatation vasculaire cutanée intense et prolongée dans le temps, évaluée en imagerie laser Doppler.

L'étude a permis de démontrer que l'association d'un inhibiteur de PDE 5 per os et d'un donneur de NO administré par iontophorèse était bien tolérée d'un point de vue systémique. A noter qu'un cas d'hypotension artérielle est néanmoins survenu.

Il avait déjà été démontré que la iontophorèse de SNP augmentait la conductance sanguine cutanée basale mais sur une très courte durée. L'étude INFLUX-VS a permis de montrer que le sildénafil à 100 mg augmentait la conductance sanguine cutanée basale, que le sildénafil à 100 mg mais pas à 50 mg, augmentait de 44 % l'amplitude de l'hyperhémie cutanée induite par la iontophorèse de SNP en termes d'aire sous la courbe (AUC). L'association d'une iontophorèse et d'un traitement per os apparaît donc comme une nouvelle possibilité thérapeutique de délivrer une molécule localement à haute concentration en association avec une molécule per os qui lui est contre-indiquée et ce avec moins d'effets secondaires systémiques.

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RESEARCH PAPER

Oral sildenafil increases skin hyperaemia induced by iontophoresis of sodium nitroprusside in healthy volunteers

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Background and purpose: Sildenafil, a specific inhibitor of phosphodiesterase 5A (PDE5A), is currently tested as a treatment for severe Raynaud's phenomenon. Here, we tested whether sildenafil, alone or combined with local sodium nitroprusside (SNP) delivered through skin iontophoresis, increased forearm cutaneous blood conductance in healthy volunteers, and to assess how well this combination was tolerated.

Experimental approach: Ten healthy volunteers were enrolled. Variations in cutaneous vascular conductance (CVC) following oral administration of 50 or 100 mg of sildenafil with or without SNP iontophoresis were expressed as a percentage of maximal CVC, and were monitored using laser Doppler imaging. SNP iontophoresis was performed on the ventral surface of the forearm, 1 h after application of lidocaine/prilocaine cream.

Key results: Sildenafil at 100 mg, but not 50 mg, increased overall responses (area under the curve) (44%) and peak responses (29%) to SNP iontophoresis. Sildenafil at 100 mg, but not 50 mg, increased baseline CVC (75%). Incidence of headache was not changed when SNP iontophoresis was combined with sildenafil. One episode of symptomatic arterial hypotension occurred in a volunteer given 50 mg sildenafil, 30 min after the beginning of SNP iontophoresis.

Conclusions and implications: Oral sildenafil at 100 mg potentiated local skin hyperaemia induced by SNP iontophoresis, with no increased incidence of headaches. The combination of oral specific PDE5A inhibitor and nitrates administered through skin iontophoresis deserves further investigation in diseases such as severe Raynaud's phenomenon, with particular attention to the incidence of arterial hypotension.

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Keywords: sildenafil; iontophoresis; sodium nitroprusside; microcirculation; ClinicalTrials.gov (NCT00710099)

Abbreviations: AUC, area under the curve; CVC, cutaneous vascular conductance; MAP, mean arterial pressure; SNP, sodium nitroprusside

Introduction

Sildenafil is a specific phosphodiesterase 5A (PDE5A) inhibitor used as a first- or second-line treatment in scleroderma patients presenting with pulmonary arterial hypertension. Most of these patients suffer from severe Raynaud's phenomenon, and 30–50% of them exhibit cutaneous finger ulcerations, despite long-term treatment with calcium channel blockers. Specific PDE5A inhibitors have been tested as a second-line treatment of severe Raynaud's phenomenon (Levien, 2006). There are a few case reports in which the

authors tested sildenafil in the treatment of fingertip ulceration in patients with secondary Raynaud's phenomenon (Colglazier *et al.*, 2005), and a recent double-blinded, randomized clinical trial showed a functional benefit in such patients (Fries *et al.*, 2005). In addition, this study showed that sildenafil increased nail-fold capillary blood flow.

Nitrates have been used for decades for the treatment of Raynaud's phenomenon (Franks, 1982). However, systemic nitrates exhibit frequent side effects related to their potency as vasodilators. Recent investigations have shown that a new topical nitroglycerin formulation may improve the severity of Raynaud's phenomenon (Chung *et al.*, 2009), strengthening the potential interest in local administration of nitrates. Iontophoresis, a non-invasive transdermal drug delivery method using a low-intensity electric current, could be an interesting alternative to ointments. Indeed, we recently showed that

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iontophoresis of sodium nitroprusside (SNP) leads to a non-axon-reflex-dependent, increased cutaneous vascular conductance (CVC) on the forearm of patients with secondary Raynaud's phenomenon (Roustit *et al.*, 2009). Moreover, some authors suggest there is potential interest in vasodilator iontophoresis as a treatment for digital ulcerations (Murray *et al.*, 2005; 2008). Indeed, long iontophoretic stimulation may be of clinical and therapeutic value with less systemic side effects.

Sildenafil is a specific PDE5A inhibitor which decreases the metabolism of cGMP. The effect of endothelial nitric oxide (NO) via cGMP is increased and leads to enhanced vasodilation. SNP is a donor of NO, which leads to an activation of guanylate cyclase and an increased production of cGMP in smooth muscle cells. The resulting vasodilation is therefore not dependent on the endothelial production of NO. Therefore, specific PDE5A inhibitors and nitrates have a synergistic pharmacodynamic action, and their co-administration significantly reduces blood pressure compared to nitrates alone (Webb *et al.*, 2000). As a consequence, the combination of oral sildenafil and nitrates is contra-indicated due to sustained arterial hypotension (Cheitlin *et al.*, 1999). Combining orally administered-specific PDE5A inhibitors with the local diffusion of a short half-life nitrate, using a controlled delivery system such as iontophoresis, could potentially reduce systemic side effects while increasing local microvascular blood flow. However, such a concept has not been demonstrated to date.

The main objective of the present study was to test whether 50 or 100 mg oral sildenafil potentiated the increase in CVC induced by local SNP iontophoresis, in the forearm of healthy volunteers. In addition, we tested whether such a combination increased the side effects related to sildenafil, with a specific focus on arterial hypotension and headaches. Cutaneous microvascular effects of the systemic administration of sildenafil, with or without SNP iontophoresis, were quantified using non-invasive laser Doppler imaging, which can routinely be used to assess skin blood flux (Cracowski *et al.*, 2006; Turner *et al.*, 2008).

Methods

Study population

This was a proof of concept study, enrolling healthy subjects recruited through local newspaper advertisements. Inclusion criteria were age of 18 years or older, no significant medical history and the absence of any severe disease (diabetes mellitus, cancer, cardiac and/or pulmonary failure, pulmonary arterial hypertension, myocardial infarction, angina pectoris). Pregnancy was excluded by urinary tests at the beginning of each visit. For all subjects, non-inclusion criteria included any allergies to local anaesthetics, cigarette smoking or dermatological disease on the arm or forearm. Grenoble Institutional Review Board (IRB n°6705) approval was obtained in May 2008, and each subject gave written informed consent before participation. The study was registered in July 2008 at ClinicalTrials.gov (NCT00710099).

Study design

This was an open-label pharmacological study. Upon arrival at the laboratory, the subjects were placed in a temperature-controlled room ($23 \pm 1^\circ\text{C}$). All subjects were sober. They were supine for the duration of the whole experiment. Heart rate and blood pressure were monitored automatically. Blood pressure was recorded every 5 min.

Each volunteer attended seven consecutive visits (V1–V7) at the Clinical Research Center (Figure 1). Each visit was separated by 7 ± 2 days. V1 was the initial enrollment visit. For the subsequent visits, the volunteers were fasted for at least 6 h before the visits. During V2, SNP iontophoresis alone was performed in one location and a control site was chosen in a different location. During V3, 50 mg sildenafil alone was given orally; at V4, 50 mg sildenafil was given orally, together with SNP iontophoresis 1 h later; at V5, 100 mg sildenafil alone was given orally; and at V6, 100 mg sildenafil was given orally together with SNP iontophoresis 1 h later. V7 was the last safety clinical visit. We intentionally chose not to randomize the different sequences for safety reasons, with a 'go/no go' decision at the end of each visit, based on clinical side effect criteria: drop in humeral systolic or diastolic blood pressure of more than 30 mm Hg, heart rate higher than 120 min^{-1} , any ophthalmic symptoms or the physician's decision.

Iontophoresis protocol and laser Doppler imaging

In order to avoid axon reflex-induced hyperaemia during iontophoresis, 1 g of lidocaine/prilocaine cream (5 g tubes containing 125 mg lidocaine and 125 mg prilocaine) was applied on the iontophoresis site 1 h before starting iontophoresis, as previously described (Cracowski *et al.*, 2007; Roustit *et al.*, 2008). The initial application of lidocaine/prilocaine cream covered 1 cm^2 of skin surface. An occlusive transparent dressing covering a larger skin area was placed over the cream to enhance cutaneous diffusion. The anaesthetized area of skin was larger than the size of the local iontophoresis probe. In order to avoid interference, the sites on the forearm were at least 3 cm apart. One hour later, the lidocaine/prilocaine cream was removed with a cotton swab. Using this procedure, we were unable to detect any change in cutaneous blood flux during NaCl 0.9% iontophoresis at $20 \mu\text{A}$ during 20 min, 60 min after lidocaine/prilocaine application, in four subjects, showing that under our experimental protocol, lidocaine/prilocaine was able to inhibit any potential axon reflex response.

Before recording, the arm was immobilized with a vacuum cushion as previously described (Roustit *et al.*, 2009). The use of this cushion decreases the artefacts associated with arm movement. After the 1 h acclimatization period at room temperature ($23 \pm 1^\circ\text{C}$), baseline laser Doppler imaging data were recorded for 5 min. Iontophoresis was then performed on the ventral face of the non-dominant forearm, followed by local heating to reach maximal blood flow.

Iontophoresis probes were used together with laser Doppler imaging (Perilont System and PeriScan PIM 3 System, Perimed, Järfälla, Sweden). The laser head was placed 20 cm above the skin. The resolution was set as 'high' (1 mm step length). Laser Doppler imaging scans were taken every minute. SNP iontophoresis was performed for 20 min at $20 \mu\text{A}$

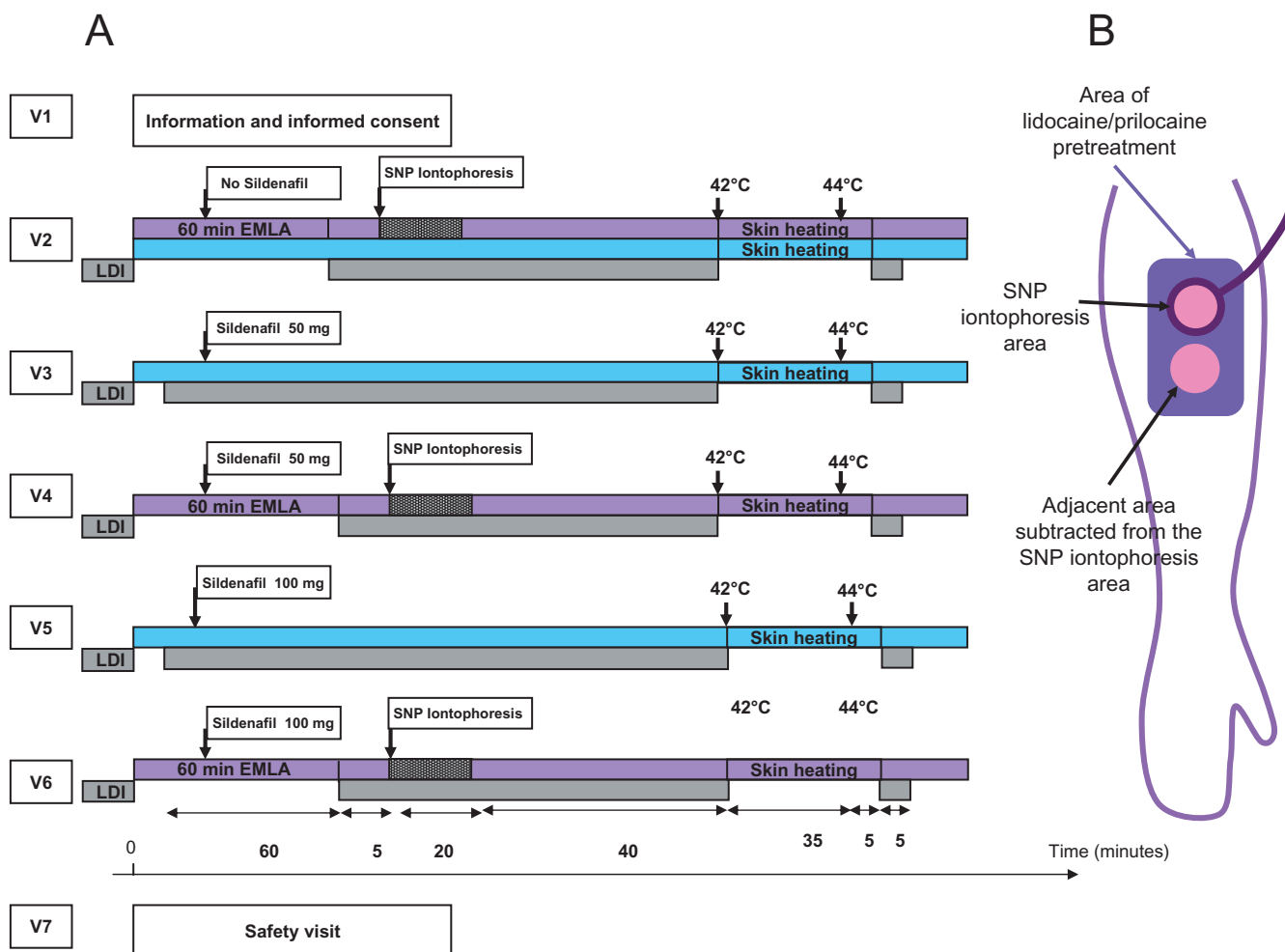


Figure 1 (A) Study protocol. Each volunteer ($n = 10$) underwent seven consecutive visits separated by 7 ± 2 days. For safety reasons, the visit sequence was not randomized, with a go/no go decision at the end of each visit based on side effects. In order to study the effect of 50 or 100 mg oral sildenafil on forearm CVC compared with no sildenafil, blue bars are used to show the sites compared. In order to study the effect of 50 mg, 100 mg oral sildenafil associated to forearm SNP iontophoresis, compared to SNP iontophoresis alone, purple bars are used to show the sites compared. In the latter case, SNP iontophoresis data were expressed as response in iontophoresis minus response in adjacent area (both subjected to surface anaesthesia with lidocaine/prilocaine) to correct for the effects of sildenafil alone, as shown (B). LDI, laser Doppler imaging; V, visit.

with 180 μ L of a 57.8 mM SNP solution (Nitrate, powder for IV injection; one vial containing 50 mg of SNP diluted in 4 mL of 0.9% NaCl solution). SNP was purchased from Serb, Paris, France. A negative charge was used for SNP iontophoresis. Our iontophoresis pads did not enable us to apply a thermoneutral temperature; however, all experiments were performed in a specific temperature-controlled room ($23 \pm 1^\circ\text{C}$) where room temperature is constantly monitored. Size of the region of interest was 1.5 cm^2 . The coefficient of variation of the peak SNP iontophoresis, calculated in eight volunteers in two measurements performed 7 days apart ± 1 , was 22%. The intraclass coefficient (ICC) of correlation was 72%. ICC values of <0.40 , $0.40\text{--}0.75$ and >0.75 represent poor, fair to good and excellent agreements, respectively (Landis and Koch, 1977). One single vial of SNP was used for each visit and discarded thereafter. The length of the data acquisition was 1 h after the start of the iontophoresis, or 2 h after administration of sildenafil alone.

At the two visits when the patient received sildenafil alone (once at 50 mg and the other at 100 mg), and at the

control site at visit V2, lidocaine/prilocaine cream was not applied; probes were located on the forearm as described above. We used the iontophoresis probes, but did not connect them to the current generator. Skin blood flux was recorded using the laser Doppler imager. Heart rate and blood pressure were monitored for 2 h after the oral intake of sildenafil.

At the end of the experiment, the skin was heated locally at all locations, for 30 min at 42°C followed by 5 min at 44°C to reach maximal skin vasodilation, using a Perimed integrated heating probe (P457, Perimed). The reason of heating first to 42°C for 30 min, and then to 44°C to obtain maximal vasodilatation was based on our practical experience in patients who exhibit a decreased capillary density like scleroderma patients who do not tolerate thermal heating to 44°C . As a consequence, we used a 42°C heating during 30 min, with only 5 min at 44°C , given the fact that 42°C does not lead to a maximal hyperaemia. Five minutes of laser Doppler imaging was recorded after the end of the skin heating sequence.

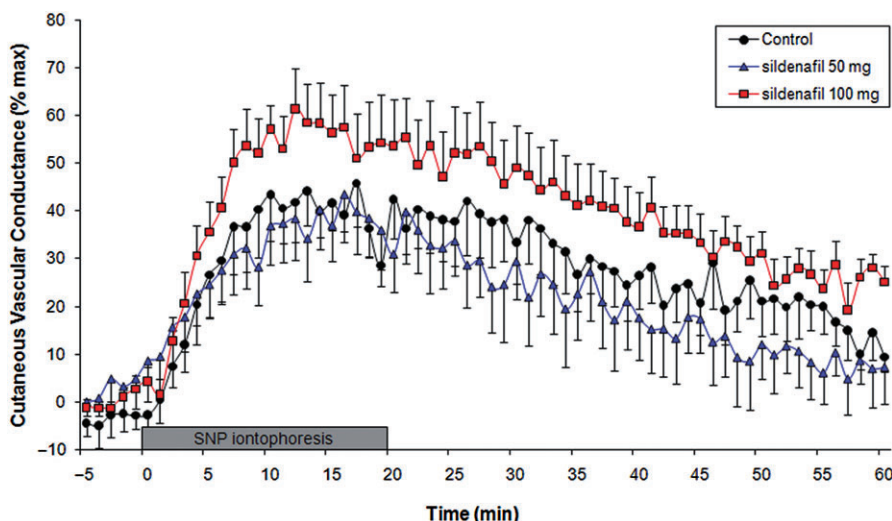


Figure 2 Effect of 50 or 100 mg oral sildenafil combined with forearm SNP iontophoresis (20 μ A during 20 min) on forearm CVC ($\text{mV}\cdot\text{mm Hg}^{-1}$). Oral sildenafil was taken 1 h before the iontophoresis onset. CVC was expressed as a percentage of maximal CVC at 44°C. Data from an adjacent control region of interest were subtracted from the iontophoresis region of interest to take into account any potential basal CVC variation induced by sildenafil alone. The increased CVC following SNP iontophoresis was significantly higher after 100 mg oral sildenafil than control ($P = 0.03$), as measured by the AUC over 60 min.

Data analysis

Data were initially expressed as CVC, which is the flux in perfusion units (PU) divided by the mean arterial pressure (MAP) in mm Hg. Subsequently, all CVC data were expressed as a percentage of maximal vasodilatation (44°C thermal plateau) (Cracowski *et al.*, 2006; Salvat-Melis *et al.*, 2006; Roustit *et al.*, 2008) and area under the curve (AUC) for SNP iontophoresis, the latter being calculated over 60 min. Following data recording, a region of interest was chosen underneath the probes on the laser Doppler scans, and flux data were used for data analysis. Data from an adjacent area (control) were subtracted from those of the iontophoresis region of interest to take into account any potential basal variation in CVC induced by sildenafil alone, and data were therefore expressed as values from the iontophoresis region of interest minus values from the control area (Figure 1B).

Quantitative data are expressed as mean and standard deviation. Qualitative data are expressed as numerical values and percentage in parentheses. Quantitative data were analysed by ANOVA for repeated measures, and with 2×2 *t*-tests for paired analyses, corrected by Bonferroni's correction, with each subject serving as his/her own control. *P* values less than 0.05 were considered statistically significant. The number of subjects, 10, was based on the hypothesis that sildenafil would induce a 25% increase in the peak hyperaemic response to SNP iontophoresis, with previous unpublished data showing that the mean SNP induced peak was $50 \pm 25\%$, with $\alpha = 0.05$, a power of 80% and a unilateral test (JD Elashoff; nQuery Advisor for Windows v 6.01, Statistical Solutions, Saugus, MA, USA).

Materials

Sildenafil, a specific PDE5A inhibitor, was purchased from Pfizer France, Paris, France. SNP, a nitrate leading to soluble

guanylyl cyclase activation through NO release, was purchased from Serb, Paris, France.

Results

Baseline characteristics

The demographic and clinical characteristics of the 10 subjects included in this study were: six females and four males, mean age 22.9 years (SD 5.3), body mass index $21.4 \text{ kg}\cdot\text{m}^{-2}$ (2.8), mean systolic arterial pressure at the beginning of the study = 117.8 mm Hg (SD 13.9), mean diastolic arterial pressure = 70.6 mm Hg (SD 10.3).

Effect of oral 50 and 100 mg sildenafil on thermal hyperaemia

Oral sildenafil did not modify the amplitude of heat (44°C)-induced hyperaemia 2 h after intake ($3.15 \pm 1.2 \text{ PU}\cdot\text{mm Hg}^{-1}$ without sildenafil, $3.27 \pm 1 \text{ PU}\cdot\text{mm Hg}^{-1}$ after 50 mg sildenafil and $3.3 \pm 1.2 \text{ PU}\cdot\text{mm Hg}^{-1}$ after 100 mg sildenafil, NS).

Main objective: effect of oral sildenafil at 50 and 100 mg on SNP iontophoresis-induced hyperaemia

The higher dose of sildenafil (100 mg), but not the lower dose (50 mg), increased the overall response to SNP iontophoresis. The corresponding AUC values were $101\ 100 \pm 70\ 415\% \text{ CVC}_{\text{max-s}}$ without sildenafil, $95\ 212 \pm 62\ 119\% \text{ CVC}_{\text{max-s}}$ after 50 mg sildenafil and $146\ 285 \pm 63\ 315\% \text{ CVC}_{\text{max-s}}$ after 100 mg sildenafil ($P = 0.03$ for 100 mg vs. control; Figure 2). Similarly, 100 mg, but not 50 mg, sildenafil increased the peak response to SNP iontophoresis (peak CVC $49.5 \pm 18.9\% \text{ CVC}_{\text{max}}$ without sildenafil, $46.6 \pm 20.3\% \text{ CVC}_{\text{max}}$ after 50 mg sildenafil and $63.9 \pm 24.4\% \text{ CVC}_{\text{max}}$ after 100 mg sildenafil; *P*

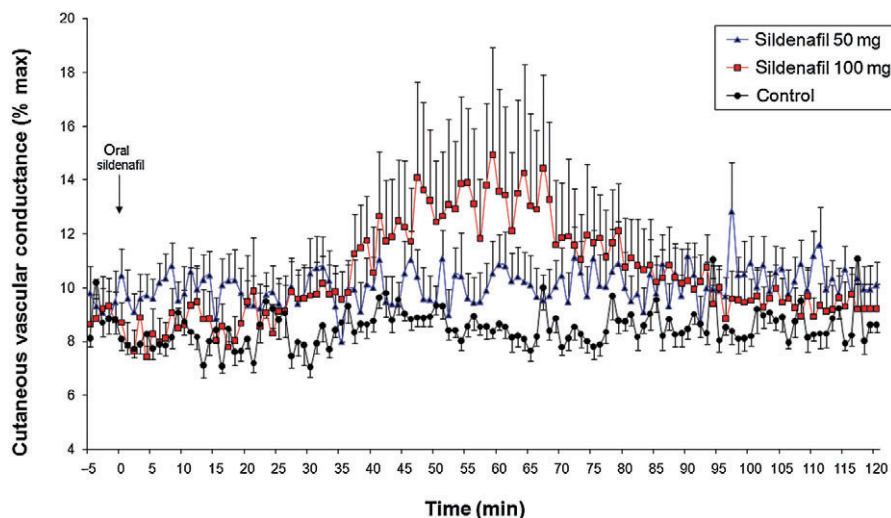


Figure 3 Effect of 50 or 100 mg oral sildenafil on forearm CVC ($\text{mV}\cdot\text{mm Hg}^{-1}$). CVC was expressed as a percentage of maximal CVC at 44°C .

= 0.05 for 100 mg vs. without control). Time to peak of SNP iontophoresis was unchanged after sildenafil (17.2 ± 8.1 min without sildenafil, 20.3 ± 9.1 min after 50 mg sildenafil and 17.3 ± 7.4 min after 100 mg sildenafil). Finally, residual flux at 60 min remained higher under sildenafil at 100 mg ($13.2 \pm 12.7\%$ CVC_{max} without sildenafil, $7.6 \pm 20.2\%$ CVC_{max} after 50 mg sildenafil and $25.5 \pm 10.9\%$ CVC_{max} after 100 mg sildenafil; $P = 0.05$ vs. without sildenafil).

Effect of oral 50 and 100 mg sildenafil given alone on baseline CVC

Sildenafil at 100 mg, but not at 50 mg, increased baseline CVC (peak CVC $8.5 \pm 1\%$ CVC_{max} without sildenafil, $10.5 \pm 3.5\%$ CVC_{max} after 50 mg sildenafil and $14.9 \pm 10.2\%$ CVC_{max} after 100 mg sildenafil; $P = 0.03$ for 100 mg vs. without sildenafil; Figure 3). Note that the peak increase in CVC with sildenafil alone occurred at 60 ± 0.8 min.

Side effects

No serious side effects occurred. We were able to give 100 mg sildenafil to all subjects. While sildenafil decreased MAP 1 h after oral intake, no individual variation in MAP was observed during the 20 min of SNP cutaneous iontophoresis, with or without sildenafil (Figure 4). At V4 (sildenafil 50 mg plus SNP iontophoresis), 10 min after the end of SNP iontophoresis, one woman exhibited pallor and dizziness associated with a 10 mm Hg drop in MAP from baseline (systolic/diastolic arterial pressure at 100/52 mm Hg at baseline, at 85/48 mm Hg during the symptoms). Her glycaemia was normal. She recovered after infusion of 100 mL NaCl 0.9%, exhibited no further side effect during the next 2 h and was able to leave the Clinical Research Center. The safety committee considered this event as not serious, but possibly related to the combination of sildenafil plus SNP iontophoresis. With her approval, she returned for the V5 and V6 (sildenafil 100 mg without and with SNP iontophoresis, respectively), where she exhibited no side effects.

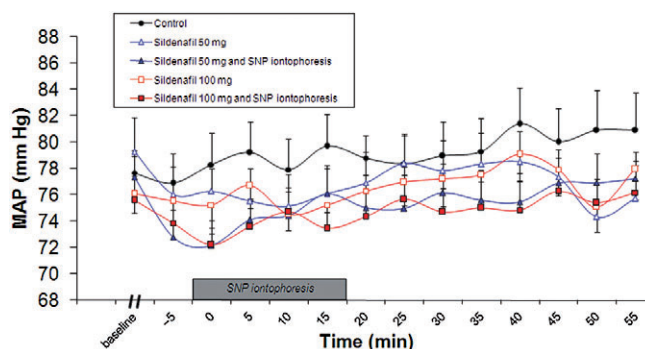


Figure 4 Effect of 50 or 100 mg oral sildenafil with or without forearm SNP iontophoresis on MAP. Oral sildenafil was taken 1 h before the iontophoresis onset. Baseline refers to MAP before sildenafil was taken. Data from baseline to SNP iontophoresis are not shown for clarity.

No headaches occurred during V2 (SNP iontophoresis alone). In contrast, four, three, three and three volunteers reported headaches at V3–V6, respectively (NS between sildenafil groups). Paracetamol (1 g orally) was taken by one, three, two and two volunteers, respectively (NS). No ophthalmic symptoms were reported.

Discussion and conclusions

We have shown that 100 mg oral sildenafil potentiated the increase in cutaneous blood conductance induced by local SNP iontophoresis, in the forearm of healthy volunteers. This pharmacodynamic potentiation occurred without an increased frequency of headaches compared with sildenafil alone. Symptomatic arterial hypotension occurred in one subject receiving 50 mg sildenafil 30 min after skin SNP iontophoresis.

Microvascular function can routinely be studied in human skin using non-invasive laser Doppler imaging (Turner *et al.*, 2008), and thermal hyperaemia is commonly used as a reference of maximal vasodilatation (Cracowski *et al.*, 2006), such

as in the present study. In our study, 50 and 100 mg of sildenafil did not alter maximal CVC 2 h after drug intake. Indeed, such results compare with those of Gooding *et al.* (2006) who showed that maximal thermal hyperaemia was unchanged following 100 mg sildenafil taken orally. However, this treatment did increase cutaneous flux 30 min after the end of the local heating, that is, sildenafil increased the duration of the hyperaemia due to local heating, but not its amplitude. We chose to provide data as CVC rather than flux to allow for potential variations in arterial blood pressure, and we further normalized conductance over the maximal hyperaemia, as this is less variable than expressing data as a percentage of baseline (Cracowski *et al.*, 2006).

Topical delivery of SNP via iontophoresis, in combination with oral sildenafil, could be considered as an alternative method of administration to increase SNP concentration locally, thus avoiding the major systemic adverse effects of both drugs. In this study, SNP iontophoresis was performed 1 h after oral intake of sildenafil, in order to match the maximal sildenafil plasma concentration, which is close to 1 h (Wright, 2006). Sildenafil absorption decreases during meals, and for this reason we chose to study fasting volunteers, in order to decrease the variability. Iontophoresis of SNP is a commonly used technique to assess non-endothelium-dependent skin microvascular function. SNP is a pro-drug, releasing NO through both enzymatic and non-enzymatic pathways, which leads to an activation of guanylate cyclase and an increased production of cGMP in smooth muscle cells. When infused intravenously, SNP has an onset of action within 30 s, with a peak effect occurring within 2 min. In our study, the effect of SNP, using iontophoresis, was delayed compared to that observed during intravenous infusion, with a peak close to 10 min. The more plausible reason for the observed potentiation of SNP increase in CVC by sildenafil is their synergistic pharmacodynamic action. However, we cannot rule out the possibility that sildenafil decreased skin resistance. Such a phenomenon would enable an increased iontophoretic SNP flux through a clearly different mechanism. As shown by Ramsay *et al.* (2002), variability in skin resistance was shown to increase the variability of iontophoresis, with high skin resistance being associated with lower vasodilation. However, in terms of therapy, the result would be similar, as it would tend to increase the vasodilator response to SNP, although through another mechanism.

Iontophoresis can cause non-specific axon reflex vasodilation. Lidocaine/prilocaine cream, applied for 1 h prior to iontophoresis, was therefore used in order to avoid such an axon reflex response during SNP iontophoresis (Cracowski *et al.*, 2007). Indeed, using lidocaine/prilocaine cream as an anaesthetic inhibits the trigger for axon reflex (C fibre action potentials). As a consequence, the effect observed is not associated with a non-specific axon reflex, but with the effect of the vasodilating substances themselves. In addition, SNP iontophoresis data were expressed as values from the iontophoresis region of interest minus the value from the adjacent region of interest to correct for any effect of oral sildenafil. The adjacent region of interest was within the anaesthetized zone. Local thermal equilibrium might have been slightly different from that within the chamber, and thus this 'adjacent area' was not

perfect. However, the effects of sildenafil alone, as shown in Figure 3, were minimal in comparison with those of SNP iontophoresis. This means that the potentiation observed after 100 mg sildenafil was not related to the baseline effect of sildenafil.

Oral sildenafil at 100 mg, but not at 50 mg, increased baseline CVC in the forearm. However, with a sample size of 10 volunteers, the study may have been under-powered to detect any effect with 50 mg. Indeed, such a short-term microvascular effect on skin vasculature is well known, as 50 mg oral sildenafil increased resting and post-ischaemic cutaneous capillary circulation in patients with coronary artery disease 1 h later (by 47%) (Park *et al.*, 2004). In addition, mean nail-fold capillary flow velocity was increased by more than 400% in patients treated with 50 mg sildenafil twice daily for 4 weeks (Fries *et al.*, 2005). Sildenafil improved the clinical condition and the anemometric flow velocity in nail-fold capillaries of patients with non-scleroderma Raynaud's phenomenon (Fries *et al.*, 2005), and was associated with thermographic and symptomatic improvement in patients with systemic sclerosis and Raynaud's phenomenon (Kumar *et al.*, 2006) or in severe digital ischaemia (Kumana *et al.*, 2004). In some series, the effects of sildenafil on Raynaud's phenomenon or digital ulcers in systemic sclerosis were spectacular and appeared within a few weeks (Gore and Silver, 2005). Regarding the side effects, headache, facial flushing and nasal congestion are the most common adverse events observed following oral administration. Headaches after sildenafil occurred as expected, but were not increased by SNP iontophoresis. Decreased MAP was observed, as expected, 1 h after oral administration of sildenafil (Figure 4), without any significant additional effect of SNP iontophoresis. However, the MAP drop associated with dizziness and pallor that occurred 10 min after the end of SNP iontophoresis (i.e. 30 min after the start of SNP iontophoresis), in a young fasting woman, was possibly related to a pharmacodynamic interaction, but the cause was uncertain given the discrepancy between the 30 min delay and the short half-life of SNP when given intravenously (a few minutes). Re-challenge (100 mg sildenafil plus SNP iontophoresis) on a different day was negative. However, such a side effect should be considered as being potentially related to the association, and merits further safety studies.

In conclusion, 100 mg oral sildenafil potentiated local skin hyperaemia induced by iontophoresis of SNP, with no increased incidence of headaches. The combination of oral specific PDE5A inhibitors and nitrates administered through skin iontophoresis should be further investigated in diseases such as severe Raynaud's phenomenon. However, further studies are required to determine the incidence of arterial hypotension induced by this combination.

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Conflicts of interest

Dr Jean-Luc Cracowski has received grant support from Pfizer for another study. The other authors report no conflict of interest.

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2.2 2^{ème} étude ancillaire d'INFLUX VS: effet du sildénafil per os sur l'épreuve d'hyperhémie post-occlusive.

Le LDI a permis l'étude de zones d'intérêt cutanées au niveau de l'avant-bras chez le volontaire sain, topographie de réalisation des iontophorèses de SNP. Dans le cadre de ce protocole complexe, nous avons effectué une étude ancillaire. L'objectif était d'étudier l'effet du sildénafil à 50 et 100 mg en prise unique sur l'hyperhémie post occlusive digitale. La fluxmétrie était nécessaire pour la réalisation des tests de chauffage à 42°C pendant 30 minutes puis 44°C pendant 5 minutes sur les doigts, sites inaccessibles aux sondes de chauffage trop volumineuses utilisées pour le LDI. La fluxmétrie a donc également été réalisée sur la pulpe et le dos des doigts. Ces données ont permis d'étudier l'effet du sildénafil 2 heures après son administration per os sur l'hyperhémie post occlusive de 5 minutes et les épreuves de chauffages. L'administration du sildénafil per os ne modifie pas le plateau de vasodilatation vasculaire cutanée maximale, en accord avec les données d'autres études (Gooding *et al.*, 2006). Elle induit par contre une durée de vasodilatation maximale post chauffage prolongée (Gooding *et al.*, 2006). L'étude INFLUX VS montre que le sildénafil à 100 mg augmente le pic de PORH, exprimé en pourcentage de baseline, de 746% (95% CI 447–1044) versus 484% (95% CI 354–613) avec 50 mg de sildénafil, et 468% (95% CI 347–588) sans sildenafil ($p = 0.03$ for 100 mg versus 50 mg et le contrôle). Le sildénafil oral à 50 mg augmente l'AUC de la PORH au niveau de l'avant-bras par rapport à la zone contrôle: 4568 PU.sec (95% CI: 2252–6883) avec 50 mg de sildénafil versus 1030 (95% CI 737–1322) sans sildénafil ($p = 0.006$). De la même manière, 100 mg de sildénafil augmente l'AUC presque significativement ($p = 0.07$). En conclusion, le sildénafil per os augmente l'hyperhémie cutanée après un temps d'ischémie. Ces résultats sont en faveur de l'implication du NO comme médiateur impliqué dans l'hyperhémie post occlusive.

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Effect of Oral Sildenafil on Skin Postocclusive Reactive Hyperemia in Healthy Volunteers

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ABSTRACT

Objective: Sildenafil is a type 5 phosphodiesterase inhibitor that has a theoretical ability to increase hyperemia following a short bout of ischemia. We tested if oral sildenafil increases skin PORH in healthy volunteers.

Methods: We assessed forearm skin PORH (occlusion of blood flow for five minutes) in ten healthy volunteers 120 minutes following the oral administration of 50 or 100 mg of sildenafil. Cutaneous blood flow on the forearm was monitored using LDF.

Results: The PORH peak, expressed as a percentage of baseline, was clearly increased with 100 mg sildenafil: 746% (95% CI 447–1044) versus 484% (95% CI 354–613) with 50 mg sildenafil, and 468% (95% CI 347–588) without sildenafil ($p = 0.03$ for 100 mg versus 50 mg and control). Oral sildenafil at 50 mg increased the AUC of PORH on the forearm compared with control:

4568 PU.sec (95% CI: 2252–6883) with 50 mg sildenafil versus 1030 PU.sec (95% CI 737–1322) without sildenafil ($p = 0.006$). Likewise, 100 mg sildenafil increased the AUC (5271 PU.sec (95% CI –81–10,623), albeit bordering on significance ($p = 0.07$). Neither dose increased maximal LTH.

Conclusions: Acute sildenafil administration at 50 and 100 mg enhances skin hyperemia following a short bout of ischemia.

Key words: sildenafil, nitric oxide, skin blood flow, microcirculation, postocclusive reactive hyperemia

Abbreviations used: AUC, area under the curve; CVC, cutaneous vascular conductance; cGMP, cyclic guanosine monophosphate; EDHF, endothelium-derived hyperpolarizing factor; LDF, laser Doppler flowmetry; L-NAME, N^G-nitro-L-arginine methyl ester; LTH, local thermal hyperemia; NO, nitric oxide; NOS, nitric oxide synthase; NS, not significant; PDE, phosphodiesterase; PK%BL, peak as a percentage of baseline; PORH, postocclusive reactive hyperemia.

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INTRODUCTION

Sildenafil is a type 5 PDE inhibitor, which decreases the metabolism of cGMP. The effect of endothelial NO on cGMP is increased leading to enhanced vasodilatation. Consequently, sildenafil could theoretically increase hyperemia following a short bout of ischemia. If confirmed, this ability could be useful in acute skin ischemia-reperfusion diseases, such as Raynaud's phenomenon.

In humans, microvascular function in the skin can be routinely studied using non-invasive LDF coupled to reactivity tests such as PORH and thermal hyperemia [3]. The mediators involved in these tests differ. The late plateau of LTH is mostly NO-dependent [8], whereas PORH is primarily dependent on EDHF [6], although there remains a controversy as to whether PORH depends on endothelial

NO release or not [7,12]. Some authors suggest that cyclooxygenase inhibition is required to reveal the PORH nitric oxide dependence [7].

Oral sildenafil had no effect on maximal skin hyperemia induced by local heating, but increases the duration of hyperemia [5]. However, no data are available describing its effect on skin ischemic hyperemia following brief arterial occlusion. We therefore tested whether 50 and/or 100 mg sildenafil increase(s) skin PORH in healthy volunteers.

MATERIAL AND METHODS

Study Population

This was a predefined ancillary study of a more complex proof of concept study [1]. Healthy subjects were recruited through local newspaper advertisements. During the main

study visits, the same volunteers participated in the ancillary study, which had a different principal objective and used different measurement devices. Inclusion criteria comprised age 18 years or more, no significant medical history, and no severe disease (diabetes mellitus, cancer, cardiac and/or pulmonary failure, pulmonary arterial hypertension, myocardial infarction, angina pectoris). For all subjects, non-inclusion criteria included any allergies to local anesthetics, dermatological disease on the arm or forearm, and smoking. Grenoble Institutional Review Board (IRB no. 6705) approved the study in May 2008 and each subject gave written informed consent before participation. The study was registered in July 2008 with clinicaltrials.gov (NCT00710099).

Study Design

This was an open label pharmacology study. Upon arrival at the laboratory, subjects were accommodated in a temperature-controlled room ($23 \pm 1^\circ\text{C}$). All subjects were sober. Sites were chosen on the ventral side of the right upper forearm, where the laser probes were placed. After a 30-minute acclimatization period and a five-minute baseline recording, PORH was performed followed by LTH (day 0). This sequence was repeated one week later, one hour after the oral administration of 50 mg of sildenafil (day 7) and two weeks later, after a 100 mg dose of oral sildenafil (day 14). PORH was then performed 120 minutes after the administration of sildenafil. The subjects remained supine for the whole duration of the experiment. Heart frequency and brachial blood pressure taken on the contralateral arm were monitored automatically every five minutes until three hours after administration of sildenafil.

Laser Doppler Measurements

To reduce artifacts associated with arm movements, throughout measurements, the arm was immobilized with a vacuum cushion as previously described [10]. Cutaneous blood flow was measured on the forearm using LDF (Periflux System 5000; Perimed, Järfälla, Sweden) with an integrated local heater (Probe 457; Perimed, Järfälla, Sweden). Laser wavelength was 780 nm and processing frequency bandwidth was 20 Hz to 15 kHz. Sampling frequency was 32 Hz. Three hours of measurements were recorded after each sildenafil administration.

PORH and Local Thermal Hyperemia Assessed with LDF

Two hours after sildenafil administration and following baseline recordings, PORH was performed. Blood flow was occluded for five minutes by inflating a cuff on the right upper arm to 50 mmHg above the systolic blood pressure. At cuff release, PORH was recorded for five minutes as previously described [10].

Five minutes later, LTH assessment using LDF was started by local heating at 42°C for 30 minutes followed by five minutes at 44°C to achieve maximal blood flow, as previously described [3,10,11].

Data Analysis

Data were digitized and analyzed offline using signal processing software (PeriSoft 2.5.5, Perimed, Järfälla, Sweden) for LDF measurements. Data were expressed as CVC, which is the flux in mV divided by the mean arterial pressure in mmHg. The use of CVC is a physiological approach that takes into account differences and variations in blood pressure [3]. PORH data were expressed as peak CVC, expressed as a percentage of baseline, and AUC expressed in PU.sec (baseline flux being deleted from the raw AUC). The kinetics of the response was determined by the time to peak (time from cuff release to peak hyperemia, in seconds).

Statistical Analysis

Qualitative data were expressed as numerical values with the percentage in parenthesis. Quantitative data are expressed as mean and 95% mean confidence interval in parenthesis, and were analyzed with ANOVA followed by a unilateral Student's paired *t*-test. *p*-Values <0.05 , corrected using Bonferroni correction, were considered statistically significant. The number of subjects was based on the hypothesis that oral sildenafil would induce a 50% increase in maximal PORH response, using a single-paired group *t*-test, with a 0.025 two-sided difference level and 80% power (nQuery Advisor[®] for Windows v 7; Statistical Solutions, Cork, Ireland).

RESULTS

Baseline Characteristics

The demographic and clinical characteristics of the 10 subjects included in this study were: six females and four males, age = 20 years (17–23); body mass index = 20.3 kg/m^2 (17.9–22.6); systolic arterial pressure at study entry = 119 mmHg (108.1–129.8); diastolic arterial pressure = 70 mmHg (59.3–80.6).

Main Objective: Effect of Oral Sildenafil on PORH

The 100-mg dose of sildenafil clearly increased the PORH peak expressed as a percentage of baseline: 746% (447–1044) versus 484% (354–613) with 50 mg sildenafil and 468% (347–588) without ($p = 0.03$ for 100 mg versus 50 mg and control; 95% CI of differences 29–525 for 100 mg versus control) (Figure 1).

Oral sildenafil at 50 mg increased the AUC of PORH on the forearm compared with control: 4568 PU.sec (2252–6883) with sildenafil 50 mg versus 1030 PU.sec (737–1322)

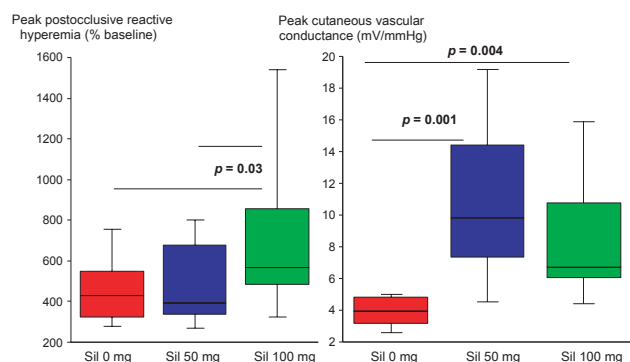


Figure 1. Peak PORH expressed as a percentage of baseline (left) and CVC (mV/mmHg, right) on the forearm without sildenafil (red box), with 50 mg sildenafil (blue box), and 100 mg sildenafil (green box).

without sildenafil ($p = 0.006$; 95% CI of differences 1329–5747). Similarly, 100 mg sildenafil increased the AUC (5271 PU.sec (−81–10,623) compared with control, although significance was not quite attained ($p = 0.07$; 95% CI of differences −999–9481).

Time to peak, in seconds, was 41 (0–84) without sildenafil, 42 (21–63) with 50 mg sildenafil, and 60 (16–103) with 100 mg sildenafil (NS).

Effect of 50 and 100 mg Oral Sildenafil on Baseline CVC

Oral sildenafil had no significant effect on mean arterial pressure. With 50 mg sildenafil, mean arterial pressure was 78.5 mmHg (75.8–81.2) at baseline and 75.1 mmHg (67.3–82.9) two hours after oral intake. With 100 mg sildenafil, mean arterial pressure was 75 mmHg (71.72–78.28) at baseline and 77.5 mmHg (70.7–84.3) two hours after oral intake.

Both doses induced a weak but significant increase in baseline CVC (0.73 (0.55–0.9) mV/mmHg without sildenafil, 1.11 (0.91–1.31) after 50 mg, and 1.13 (0.8–1.46) after 100 mg sildenafil ($p = 0.012$ for 100 mg versus without sildenafil; $p = 0.24$ for 50 mg versus without sildenafil).

Effect of 50 and 100 mg Oral Sildenafil on 44°C Hyperemia

Neither dose significantly increased 44°C induced thermal hyperemia 35 minutes after the initiation of local heating. On the forearm, CVC after 44°C hyperemia was 4.4 (3–5.7); 5.8 (3.8–7.8) and 6.1 (4.9–7.3) mV/mmHg without, with 50 and 100 mg sildenafil, respectively (NS).

Side Effects

We observed no unexpected side effects in any of the subjects enrolled. However, none, four, and three subjects reported headaches during the control, 50 mg, and 100 mg sildenafil visits, respectively, requiring 1000 mg oral

acetaminophen in none, one, and two subjects, respectively. All patients were able to receive 100 mg of sildenafil.

DISCUSSION

Our study shows that oral sildenafil enhances skin PORH. We used PORH and LTH as reactivity tests for assessment of skin microvascular function. We chose LDF as this technique has a good temporal resolution in contrast to laser Doppler imaging that has better spatial resolution. PORH reproducibility remained correct under our experimental conditions [10,11]. The two dosages of sildenafil tested are those commonly used in clinical practice for erectile dysfunction. Due to the experimental design of the main study [1], for safety reasons, this ancillary study was not randomized placebo double-blinded. This is unlikely to bias our results as the main endpoint was objective (maximal value of PORH and AUC). Sildenafil altered baseline flux. Therefore, we showed peak PORH (expressed as a percentage of baseline) and raw AUC (expressed as PU.sec) that had the advantage of baseline subtraction. Sildenafil increased the amplitude and duration of the postocclusive hyperemia at 50 and 100 mg, as reflected by the increased AUC, with an effect on the peak observed only at 100 mg. Indeed, expressing peak PORH as a percentage of baseline gives baseline values a higher impact than AUC, where baseline is only subtracted. We did not calculate the time required to come back to baseline, as this parameter is difficult to determine accurately and is not reproducible. However, AUC incorporates this parameter, while peak flow is transient and only partly affects AUC.

Sildenafil improved NO-dependent endothelial function in patients with heart failure or diabetes [2,9], but not in smokers [4]. This discrepancy can be explained by the fact that the studies used different methodologies and focused on different vascular beds. We recently showed that sildenafil increases baseline CVC in healthy subjects [1]. Gooding *et al.* previously showed that NO is a contributor to maximum skin hyperemia in response to local heating through an attenuation of maximal hyperemia by L-NAME, an NOS competitive inhibitor, when delivered by microdialysis for 30 minutes [5]. Sildenafil at 100 mg in healthy subjects did not alter maximal hyperemia [5], but the resolution was modified as the hyperemia was prolonged in the presence of sildenafil [5]. In our study, thermal hyperemia was not altered, but the small size of the study was prone to type 2 error. Dishy *et al.* showed that in chronic male smokers sildenafil had no significant effect on postischemic hyperemia, explored with strain gauge plethysmography [4]. However, the vascular bed studied by plethysmography is mostly forearm muscle, in contrast to our skin investigation. Furthermore, the dose of sildenafil was 50 mg and a potential dose effect with 100 mg was not tested.

There has been much debate over the physiological mediators implicated in skin postischemic hyperemia. The response is complex, and involves EDHFs and axon reflexes [6]. While NO synthase inhibition failed to demonstrate a role of NO, such a role was demonstrated when cyclooxygenase was pharmacologically blocked [7,12]. Our results may support the concept that NO is a mediator involved in skin postocclusive hyperemia.

In conclusion, we show that oral sildenafil enhances skin hyperemia following a short bout of ischemia. The therapeutic implications should be further investigated in conditions involving skin ischemia-reperfusion, such as Raynaud's phenomenon.

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CONFLICT OF INTERESTS

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3 DEUXIEME PARTIE : Screening de molécules candidates à la iontophorèse cutanée chez le rat : étude INFLUX-RAT

Exceptée la iontophorèse de SNP et d'acétylcholine pour des tests physiologiques, il n'existait pas d'autres molécules à action vasodilatatrice puissante ayant été testées en iontophorèse cutanée chez l'homme. Nous avons donc réalisé dans le cadre d'un screening systématique des iontophorèses chez l'animal (rat). Les médicaments testés ont été les molécules évaluées dans les trois voies thérapeutiques développées dans le traitement d'une des atteintes vasculaires de la ScS, l'HTAP, à savoir : la voie du NO, la voie des antagonistes de l'endothéline et la voie des prostacyclines.

Tableau 1: Résumé des principales caractéristiques physico-chimiques des molécules testées chez le rat. (issu de <http://www.chemicalize.org/>)

Voie explorée	Molécules	MW (g/mol)	Isoelectric point ou pKa	pH de la solution testée
Prostacycline	Epoprostenol	352.5	4.5	10.5 et 6,5
	Treprostinil	390.5	4.5	6,5
	Iloprost	478.6	6.46	6
Endotheline-1	Bosentan	573.6	2.64	7
	Sitaxentan	454.9	4.57	6
NO	Sildenafil	747.6	8.45	5.5 et 3.5
	SNP	215.9	1.49	

La démarche globale a été de tester les médicaments de ces trois grandes classes thérapeutiques. En ce qui concerne la voie des **antagonistes de l'endothéline**, le bosentan a été obtenu sous forme de poudre et a été dissous en tenant compte du coefficient de solubilité et du pH. La iontophorèse en anodal et cathodal pendant 20 minutes à 100 μ A n'a pas mis en évidence de vasodilatation cutanée et nous concluons à un échec de la iontophorèse de bosentan. Le sitaxentan était également sous forme de poudre. Après une transformation via une solubilisation, la iontophorèse cathodale de sitaxentan n'a pas permis de mettre en évidence vasodilatation cutanée. Par contre, une iontophorèse de sitaxentan après injection intra artérielle d'endothéline a permis de mettre en évidence une réversion partielle de l'effet de l'endothéline-1. Ces résultats suggèrent que le sitaxentan passe au travers de la peau en iontophorèse, mais que le tonus vasculaire basal cutané n'est pas endothéline-1 dépendent. Malgré ces résultats négatifs, nous avons testé dans le cadre du protocole INFLUX SIT VS la iontophorèse de sitaxentan chez 6 volontaires sains. Les résultats n'ont pas mis en évidence de vasodilatation vasculaire cutanée et nous avons conclu à un échec de la iontophorèse de sitaxentan chez l'homme sur le paramètre effet vasodilatateur. Le retrait de la molécule commercialisée Thellin® en décembre 2010 du fait de quelques cas d'hépatotoxicités léthales, a stoppé le développement de la iontophorèse de sitaxentan chez le volontaire sain en évaluant d'autres paramètres que la vasomotricité.

En ce qui concerne **la voie du NO**, le SNP n'étant pas un bon candidat du fait de sa cinétique, le sildénafil était disponible sous forme poudre et a été dissous. Hélas, le coefficient de solubilité explique que la forme soluble à pH 5 n'était pas stable. Nous avons conclu à l'impossibilité de réaliser la iontophorèse de sildénafil.

En ce qui concerne les **prostacyclines et analogues**, les résultats animaux ont été concluants. La iontophorèse cathodale de tréprostinil (à 6.4 mM, 0.64 mM et 0.064 mM) induit une vasodilatation cutanée prolongée (AUC de 80 minutes) avec un effet concentration-dépendant. Le passage du tréprostinil avec une polarité cathodale pouvait être attendu du fait que le tréprostinil est ionisé négativement, le phénomène d'électro-répulsion favorisant sa diffusion dans le derme.

L'iloprost (à 0.2 mM, 0.02 mM et 0.002 mM) induit également une vasodilatation cutanée à la iontophorèse dans le sens cathodal à l'AUC de 80 minutes. L'époprosténol induit également une augmentation du flux sanguin cutané dans le sens cathodal. Son pH basique à 12 nous a conduits à tester l'époprosténol à un pH compatible avec une application cutanée chez l'homme (pH à 6,5) qui n'a pas induit une vasodilatation suffisante.

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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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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 anaesthetized 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

PGI₂, 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 (PGI₂) analogue used intravenously, is the only drug approved for the treatment of existing digital ulcers (Wigley *et al.*, 1992). In addition, treprostinil, a PGI₂ analogue used subcutaneously, and epoprostenol (intravenous PGI₂) 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 PGI₂ analogues could have potential for the treatment of digital ulcers. However, to our knowledge, iontophoresis of PGI₂ analogues has never been tested, either in animals or in humans.

The main objective of the present study was to test whether iontophoretically administered vasodilator drugs such as PGI₂ (epoprostenol) and PGI₂ analogues (iloprost and treprostinil) induce an increase in cutaneous blood flow in rats. As a secondary objective, we also assessed the cutaneous tolerance of PGI₂ 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 PGI₂ analogues and intravenous PGI₂ 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) (PGI₂ 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



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).

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.

Iontophoresis protocol and laser Doppler imaging (LDI)

Iontophoresis probes were used together with LDI (Perilont 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-cm² 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 μ A using both anodal and cathodal currents ($n = 8$ for each series). Iontophoresis chambers contained 180 μ L 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 ($AUC_{20\text{min}}$ or $AUC_{80\text{min}}$ 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 ($115\,669 \pm 49\,812\%$ BL.s, $P < 0.001$), iloprost 0.2 mM ($60\,180 \pm 43\,069\%$ BL.s, $P = 0.005$) and epoprostenol 0.028 mM pH 12 ($42\,865 \pm 24\,922\%$ BL.s, $P = 0.0018$) induced an increase in $AUC_{20\text{min}}$ significantly different from 0 (Figure 2A). As expected, SNP induced a significant increase in $AUC_{20\text{min}}$ ($41\,937 \pm 28\,785\%$ BL.s, $P = 0.008$). However, epoprostenol 0.028 mM pH 6.5 ($14\,557 \pm 18\,012\%$ BL.s) and NaCl ($18\,071 \pm 16\,677\%$ BL.s) did not induce a significant increase in skin blood flux, expressed as $AUC_{20\text{min}}$.

At the anode, although we observed a non-significant trend towards a small increase in $AUC_{20\text{min}}$ ($36\,792 \pm 49\,268\%$ BL.s, $P = 0.07$) for iloprost 0.2 mM, this was only significantly different from 0 for treprostinil 6.4 mM ($AUC_{20\text{min}} 24\,695 \pm 26\,339\%$ BL.s, $P = 0.048$) (Figure 2B). No increase in $AUC_{20\text{min}}$ 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 ($107\,340 \pm 42\,269\%$ BL.s, $P < 0.0001$) and iloprost 0.2 mM ($49\,484 \pm 21\,842\%$ BL.s, $P < 0.001$) induced an increase in $AUC_{20\text{min}}$ 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,

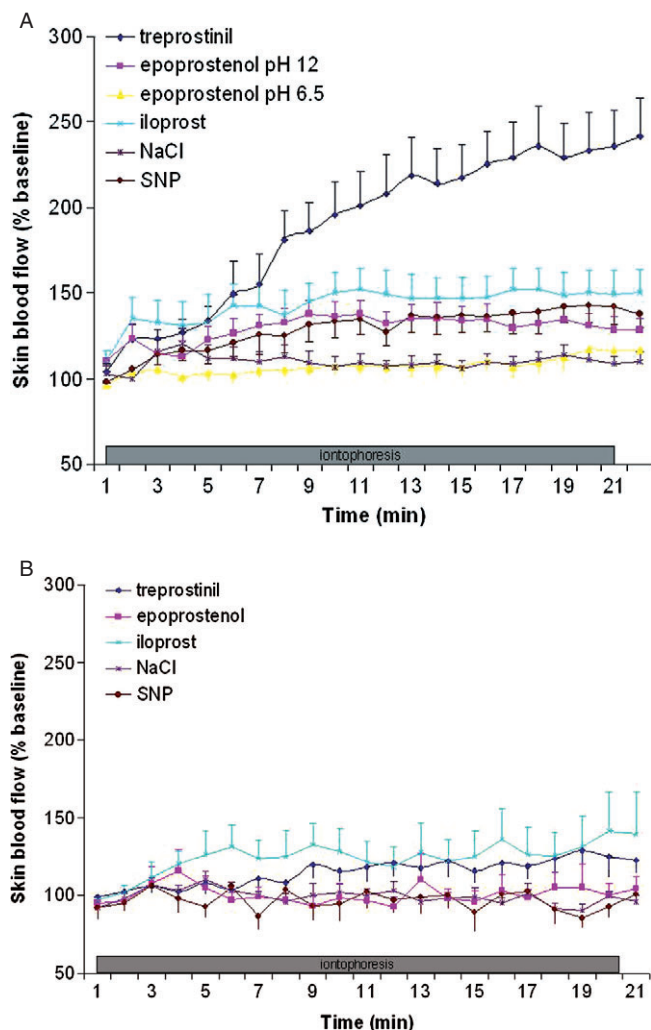


Figure 2

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 ($270\,963 \pm 117\,637\%BL.s$, $342\,568 \pm 379\,205\%BL.s$, $190\,418 \pm 108\,746\%BL.s$, respectively, NS) (Figure 4A).

Iloprost at 0.02 mM induced a significant increase in AUC_{80min} in comparison with iloprost 0.002 mM ($379\,597 \pm 196\,125\%BL.s$ vs. $96\,730\%BL.s \pm 145\,138$, respectively, $P = 0.02$) (Figure 4B). Iloprost at 0.2 mM tended to increase the AUC ($270\,126\%BL.s \pm 303\,908$) 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.

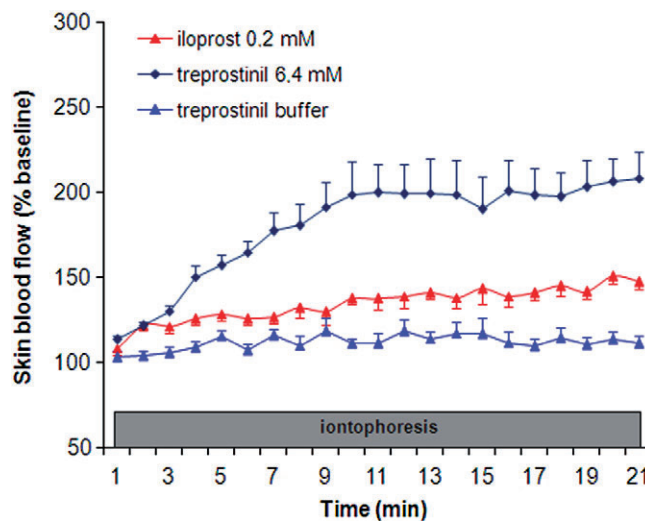


Figure 3

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 ($45 \pm 7\,K\Omega$) and iloprost 0.2 mM ($44 \pm 5\,K\Omega$) were not significantly different from that obtained with NaCl ($45 \pm 7\,K\Omega$, NS). When these solutions were diluted, skin resistances were still not different from that with NaCl: $46 \pm 14\,K\Omega$ for treprostinil 0.64 mM, $49 \pm 14\,K\Omega$ for treprostinil 0.064 mM, $51 \pm 14\,K\Omega$ for iloprost 0.02 mM and $48 \pm 8\,K\Omega$ 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 $26\,048 \pm 30\,792\%BL.s$ at 0.02 mM vs. $15\,178 \pm 22\,251\%BL.s$ 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

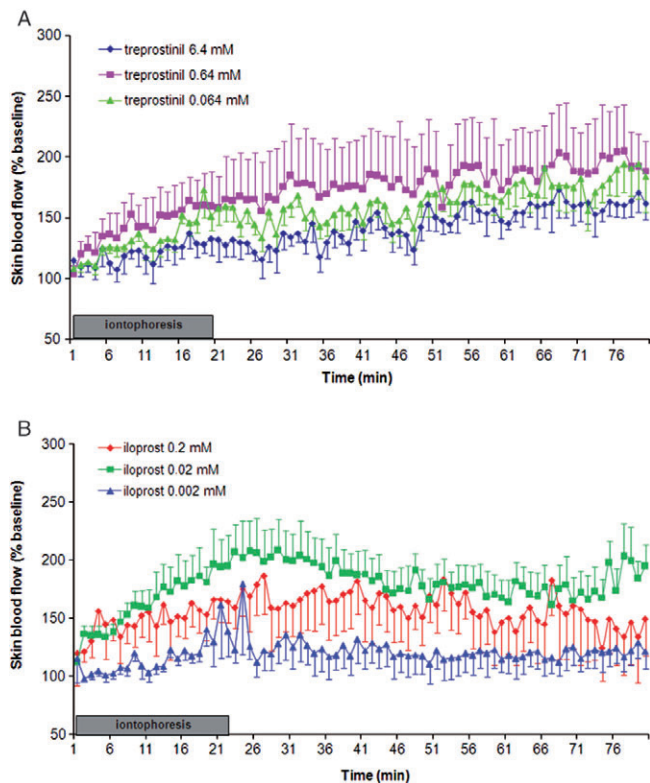


Figure 4

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.

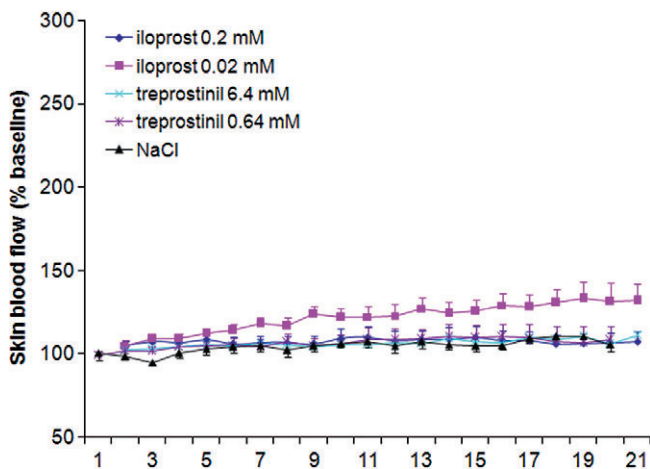


Figure 5

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

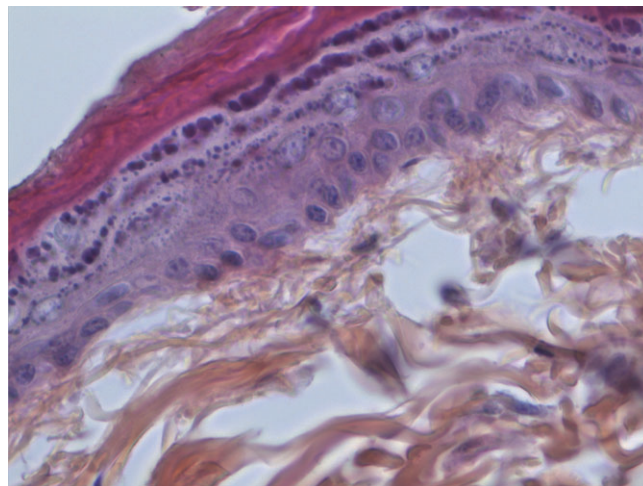


Figure 6

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 ($\times 40$).

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.

Discussion and conclusion

This is the first demonstration that cutaneous cathodal iontophoresis of treprostinil and iloprost, two PGI₂ 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 PGI₂ 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 pK_a, 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 pK_a value of 7.5. The second possibility is that the stability of PGI₂ 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 μ A 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 μ A through rat skin to that found when we used a 20- μ A 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 μ M and a maximal effect at 20 μ M). 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 PGI₂ 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 μ A 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 μ M and iloprost as low as 20 μ M. 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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Iontophoresis of endothelin receptor antagonists in rats and men

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SUMMARY

Background and purpose. The treatment of scleroderma related digital ulcers remains a therapeutic challenge. The oral endothelin receptor antagonist (ERA) bosentan is marketed as a first-line treatment but its systemic administration may induce liver toxicity. The objective of this study was to test whether iontophoresis of ERAs bosentan and sitaxentan can induce sustained local vasodilation.

Experimental approach. Bosentan and sitaxentan were delivered by cathodal and anodal iontophoresis on anaesthetized rat hindquarters without and during endothelin-1 infusion. Skin blood flow was quantified using laser Doppler imaging and cutaneous tolerance was assessed. After laboratory studies, iontophoresis of sitaxentan (20 min, 20 or 100 μ A) was performed on the forearm skin of healthy young men (n=5).

Key results. In rats neither cathodal nor anodal iontophoresis of bosentan or sitaxentan increased skin blood flux compared to NaCl. When simultaneously infusing endothelin-1, cathodal iontophoresis of sitaxentan significantly increased skin blood flux compared to NaCl (AUC_{0-20} were 44032.2 ± 12277 and 14957.5 ± 23818.8 %BL.s, respectively; $P= 0.01$). In humans, sitaxentan did not significantly increase skin blood flux as compared to NaCl. Iontophoresis of ERAs was well tolerated both in animals and humans.

Conclusions and implications. 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. However, sitaxentan does not influence basal skin microvascular tone either in rats or in humans.

Keywords: iontophoresis, sitaxentan, bosentan, endothelin, microcirculation, vasodilation.

Abbreviations:

cutaneous vascular conductance = CVC

endothelin-1 = ET-1

endothelin receptor antagonists = ERA

laser Doppler imaging = LDI

prostacyclin = PGI₂

systemic sclerosis = SSc

For Peer Review

Introduction

Systemic sclerosis (SSc) is a rare disease affecting digital microcirculation, leading to finger ulcers and in some cases to amputation (Herrick, 2000). 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 (Korn *et al.*, 2004). Elevated aminotransferase levels is the main adverse effect of bosentan, with an annual rate of 10.1%, leading to therapy discontinuation in 39% cases (Humbert *et al.*, 2007). Prostacyclin (PGI₂) analogues are used intravenously (Wigley *et al.*, 1992), 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 (Kalia *et al.*, 2004). Some authors have highlighted the potential interest of iontophoresis of vasodilating drugs as a treatment for digital ulcers in SSc (Murray *et al.*, 2005; Murray *et al.*, 2008) and previous work from our laboratory has suggested that PGI₂ analogues are appropriate candidates (Blaise *et al.*, 2011). 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.

Methods

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 (Blaise *et al.*, 2011).

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 (Harvard apparatus).

Experiment 1: Iontophoresis of bosentan, sitaxentan and NaCl (20 min, $100 \mu\text{A}$) 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 scoring (Fregert, 1981). Negative reactions were coded grade 0; weak reactions (grade 1) are characterized by 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. Arterial pressure (Powerlab, ADInstrument) was recorded through the first catheter and ET-1 (5 nmol.kg^{-1} i.a. bolus followed by $0.5 \mu\text{L.min}^{-1}$ infusion) was administered through the second catheter. Iontophoresis of bosentan, sitaxentan and NaCl (20 min, $100 \mu\text{A}$) 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 ($n=9$: 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

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2
3 Erba) paraffin-embedded and stained with hematoxylin, eosin, and safran. In order to evaluate
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5 the effect of the treatment on the skin, various features were sought, including hyperkeratosis
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7 and epidermolytic aspects, vasculitis and inflammation [the histopathologic examination
8
9 procedure have been previously detailed (Blaise *et al.*, 2011)].
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12 13 14 Skin blood flux measurement and data analysis

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16 Three 1.2 cm² circular iontophoresis electrodes (PeriIont System, Perimed, Järfälla, Sweden)
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18 containing bosentan, sitaxentan or NaCl were placed on the hairless skin of the lower
19
20 back/hind legs. Passive electrodes were placed on the back of the neck, as previously
21
22 described (Blaise *et al.*, 2011). Skin blood flux was continuously recorded with laser Doppler
23
24 imaging (LDI; PeriScan PIM 3, Perimed, Järfälla, Sweden). The laser head was placed 20 cm
25
26 above the skin. The resolution was 2 mm step length and LDI scans were taken every minute.
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30
31 In experiment 1, iontophoresis was started after a 5-min baseline (BL) recording. Skin
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33 blood flux was then recorded during iontophoresis and during the 20 min following the end of
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35 iontophoresis, and expressed as arbitrary perfusion units (PU). In order to take into account
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37 inter-individual BL variations, data were subsequently expressed as a percentage change from
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39 BL (%BL). Then, a minute by minute analysis was performed to calculate the area under the
40
41 curve during iontophoresis (AUC₀₋₂₀, in %BL.s) and during the whole recording (AUC₀₋₄₀).
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45
46 In experiment 2, the skin blood flux was recorded for 5-min under resting conditions.
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48 Iontophoresis was performed 5 min after ET-1 infusion started, time for blood flux to
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50 decrease and reach a plateau (baseline). Data were expressed as percentage change between
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52 baseline and the iontophoresis plateau (averaged over 10 min) (expressed as %BL). Then,
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54 AUC₀₋₂₀ was calculated as described above and expressed as %BL.s. In order to take into
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56 account ET-1-induced variations in blood pressure, we expressed data as cutaneous vascular
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58 conductance (CVC), i.e. flux divided by arterial pressure (in mV.mm Hg⁻¹) (Cracowski *et al.*,
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60 2006; O'Leary, 1991), to subsequently calculate AUC_{CVC 0-20} (expressed as %BL.s).

Clinical study

Study population

We recruited healthy men through the Clinical Research Centre 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 ($23\pm 1^{\circ}\text{C}$) during 1 hour for acclimatization. They remained supine for the whole experiment.

Three consecutive visits were planned for each volunteer, separated by 7 days \pm 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

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2
3 lidocaine and 125 mg prilocaine, Anesderm[®], Pierre Fabre, Boulogne, France) in order to
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5 attenuate axon reflex-induced hyperaemia (Blaise *et al.*, 2010). An occlusive transparent
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7 dressing was placed over the cream to enhance cutaneous diffusion. Each anesthetized area
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9 was larger than the area of the iontophoresis chambers.
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13 One hour after lidocaine/prilocaine application (corresponding to the acclimatization
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15 period) the cream was removed with a cotton swab. The two pre-treated skin sites were then
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17 equipped with 1.2 cm² circular iontophoresis chambers (PeriIont System, Perimed, Järfälla,
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19 Sweden) containing sitaxentan or NaCl. After a 5-min baseline recording, cathodal
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21 iontophoresis was simultaneously performed for 20 minutes with a current intensity set at 20
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23 μ A. After iontophoresis, skin blood flux was recorded for an additional 20 min. During the
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25 experiment, the two remaining skin sites were pre-treated with local anaesthetic cream for one
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27 hour. The same protocol as described above was then performed on these skin sites, the only
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29 difference being the current intensity set at 100 μ A.
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34 Skin blood flux was recorded throughout with laser-Doppler imaging (PeriScan PIM
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36 3, Perimed, Järfälla, Sweden). The resolution was 1 mm step length and LDI scans were taken
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38 every 30 seconds. Before recording, the arm was immobilized with a vacuum cushion to
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40 decrease artefacts related to arm movement. Baseline and maximal skin blood flux were
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42 averaged over 5 min. Data were subsequently expressed as a percentage change from BL
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44 (%BL) and AUC₀₋₄₀ were calculated (expressed as %BL.s).
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50 51 *Statistical analysis*

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53 Categorical data were reported as frequency and percentage and continuous data as mean and
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55 standard deviation. They were analyzed by repeated measures ANOVA, and paired t tests for
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57 2x2 comparisons. When the conditions of application of parametric tests were not respected,
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59 nonparametric tests were used (Friedman test, and Wilcoxon test for paired comparisons).
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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).

Results

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 1; Figure 1). Iontophoresis did not significantly affect blood pressure (87.5 ± 17.9 mmHg before and 90.6 ± 10.2 mmHg after iontophoresis; $P=0.7$).

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

Resting 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 2). Cathodal iontophoresis of sitaxentan significantly increased skin blood flux compared to NaCl. However, we observed no significant difference between bosentan and NaCl (Table 2; Figure 3A).

Resting 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

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3 PU, respectively ($P=0.5$). Anodal iontophoresis of bosentan or sitaxentan did not significantly
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5 increase skin blood flux compared to NaCl (Table 2; Figure 3B).
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9 10 Skin tolerance of the iontophoresis of bosentan and sitaxentan

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12 No skin side-effects were observed. In experiment 1, all the cutaneous scores were 0 both
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14 immediately after the procedure and at day 3. In experiment 2, none of the various
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16 histopathologic features listed in the Methods section were found in any of the skin biopsies.
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19 20 21 22 *Clinical study*

23 24 Study population

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26 Six male volunteers were enrolled in the study. One of them did not attend visits 2 and 3 and
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28 was excluded. The characteristics of the population were: mean age 22.8 ± 1.9 years and body
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30 mass index 22.1 ± 1.5 kg/m². Mean systolic/diastolic arterial pressure at inclusion was $115 \pm$
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32 $7.4 / 77 \pm 10.9$ mm Hg.
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36 37 38 Effect of cathodal iontophoresis of sitaxentan on skin blood flux

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40 Neither sitaxentan nor NaCl increased skin blood flux when the iontophoresis current
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42 intensity was 20 μ A (AUC_{0-40} were -2785.3 ± 35811.4 %BL.s and -3315.1 ± 42523.1 %BL.s,
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44 respectively; $P=0.69$). Cathodal iontophoresis of sitaxentan induced an inconstant increase in
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46 skin blood flux when the current intensity was set at 100 μ A (AUC_{0-40} was $289409.4 \pm$
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48 348171 %BL.s). However, a similar increase was observed with NaCl (AUC_{0-40} was 236430.4
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50 ± 348333.1 %BL.s; $P=0.22$) (Figure 4A).
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55 56 57 Tolerance

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3 No side-effect occurred. We observed no significant change in mean arterial pressure during
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5 iontophoresis of sitaxentan (Figure 4B). No squamous, vesicular or bullous lesions were
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7 observed at the end of the experiment or at the safety visit. Erythema was observed in one
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9 participant. It was localized to the area surrounding the anesthetized area and was attributed to
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11 hypersensitivity to the adhesive of the dressing. Aspartate transaminase (AST)/alanine
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13 transaminase (ALT) at visit 1 and visit 3 for each volunteer were: 34/17 and 36/14 U.I.L⁻¹,
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15 18/27 and 21/21 U.I.L⁻¹; 16/15 and 20/12 U.I.L⁻¹; 30/25 and 31/25 U.I.L⁻¹; 30/18 and 26/29
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17 U.I.L⁻¹.

21 22 23 24 **Discussion and conclusion**

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26 This study showed that in basal conditions iontophoresis of bosentan (in rats) or sitaxentan (in
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28 rats and humans) did not increase skin blood flux. However, after ET-1 infusion,
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30 iontophoresis of sitaxentan significantly increased cutaneous blood flux in rats, suggesting
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32 that the absence of effect initially observed was due to low ET-1-dependent basal skin
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34 microvascular tone rather than the absence of diffusion of the antagonist into the skin.
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36 However, no effect was seen with bosentan. Iontophoresis of ERAs was well tolerated both in
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38 animals and humans.
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43 Iontophoretic transport is influenced by several parameters, including drug
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45 concentration, molecular weight, ionization and solution pH or current strength and skin
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47 hydration and resistance (Dixit *et al.*, 2007). Bosentan and sitaxentan are small molecules
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49 with isoelectric points (pI) at 2.64 and 4.57, respectively. These chemical properties make
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51 these ERAs appropriate candidates for cathodal iontophoresis, being negatively ionized at
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53 neutral pH. However, in basal conditions, we were not able to see any effect of the
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55 iontophoresis of bosentan or sitaxentan on skin blood flux in rats (experiment 1). In order to
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57 test whether the absence of effect was related to low ET-1-dependent vascular tone in the skin
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3 of healthy rats or to the absence of iontophoretic transport into the dermis, we repeated the
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5 experiment while infusing ET-1 (experiment 2). The results of this series suggest that the
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7 cathodal iontophoresis of sitaxentan enabled the drug to be locally administered in sufficient
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9 concentration to partially reverse the sustained effect of ET-1 on the skin microcirculation.
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11 However, such an effect was not observed with bosentan, suggesting that we were unable to
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13 reach sufficient skin concentration to reverse ET-1 vasoconstriction. It is of interest that we
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15 obtained similar results when data were expressed as cutaneous vascular conductance, to take
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17 into account the effect of ET-1 on arterial blood pressure. The effect of ET-1 on blood
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19 pressure was comparable to that previously described: an initial transient decrease in blood
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21 pressure immediately after ET-1 infusion was started was followed by a prolonged increase
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26
27 (de Nucci *et al.*, 1988).

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29 The relationship between the pharmacodynamic effect of ERAs on microvascular
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31 function and their efficacy in the prevention of digital ulcers in patients at risk remains
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33 uncertain. Two groups have studied the effect of the systemic administration of bosentan on
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35 skin blood flux in patients with SSc (Hettema *et al.*, 2009; Rosato *et al.*, 2010). Hettema *et al*
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37 did not show any difference in blood flux during acetylcholine and sodium nitroprusside
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39 iontophoresis, used as pharmacological tests of microvascular endothelium-dependent and
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41 endothelium-independent function, respectively (Hettema *et al.*, 2007). Conversely, Rosato *et*
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43 *al* showed an increase in skin perfusion 8 weeks after bosentan therapy was started in SSc
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45 patients with SSc and pulmonary hypertension. In SSc patients without pulmonary
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47 hypertension however, there was no significant difference (Rosato *et al.*, 2010). These
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49 discrepancies could be due to higher ET-1-dependent vascular tone in patients with
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51 pulmonary hypertension.
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58 Insufficient diffusion of ERAs into the skin after oral administration is another
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60 hypothesis. Indeed, when administered directly into the dermis BQ-123 (an ET_A receptor

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3 antagonist) significantly increased skin blood flux in healthy volunteers (Leslie *et al.*, 2004).
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5 Interestingly, intradermal BQ-788 (an ET_B receptor antagonist) also increased blood flow
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7 (Leslie *et al.*, 2004), suggesting that both ET_A and ET_B contribute to ET-1-mediated basal
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9 microvascular tone in the human skin. On the other hand, although both ET_A and ET_B mediate
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11 vasoconstriction in rat skin (Lawrence *et al.*, 1995), injection of BQ-123 and bosentan (non
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13 specific ET_A and ET_B antagonist) did not alter basal skin blood flow in rats but reversed the
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15 effects of ET-1 (Lawrence & Brain, 1994), which is consistent with our findings.
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20 Therefore, iontophoresis probably did not allow sufficient concentrations of ERAs to
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22 be reached in human skin to be able to show any effect on the microvasculature. We did not
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24 use intradermal injections or microdialysis as these methods would be too far removed from
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26 the initial therapeutic objective, i.e. iontophoresis in physiological conditions.
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30 In healthy participants, we observed an unexpected increase in skin blood flux for both
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32 sitaxentan and NaCl when the current intensity was 100 μ A. Indeed, one of the main issues
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34 when performing iontophoresis is the non specific effect of the current itself, where the
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36 amplitude depends on the delivered electrical charge (i.e. the product of current intensity by
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38 duration of application) (Durand *et al.*, 2002a). This may explain why we did not observe any
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40 reaction when the current intensity was only 20 μ A. As a neural axon reflex has been
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42 suggested to be involved in current-induced vasodilation (Durand *et al.*, 2002a), we pre-
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44 treated the skin sites with a local anaesthetic (lidocaine/prilocaine). However, this did not
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46 abolish current-induced vasodilation in all subjects when the current intensity was 100 μ A.
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48 This could be explained by the role of prostaglandins, which are likely to be essential for the
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50 axon reflex-related vasodilatation (Durand *et al.*, 2002b), mainly through the COX-1 pathway
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52 (Tartas *et al.*, 2005).
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57 In humans the iontophoresis of sitaxentan and NaCl were both highly variable at 100
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59 μ A. Indeed, analysis of individual data reveals major current-induced vasodilation in two
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3 participants, whereas in the three other subjects we only observed a weak or even no effect at
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5 all (individual data not shown for clarity). This difference could be due to inter-individual
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7 differences in skin resistance, which was not recorded at this early stage. Nonetheless, this is
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9 unlikely to have a major impact on the conclusion of these experiments as each subject was
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11 his own control. Indeed, the iontophoresis were simultaneously performed at both sites with
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13 the same distance between electrodes.
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17 The clinical study was prematurely stopped after the manufacturer decided to
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19 discontinue all ongoing clinical trials with sitaxentan and to withdraw the commercial drug,
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21 because of several cases of fatal liver injury (Galie *et al.*, 2011). Nonetheless, despite the
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23 small sample size, there was no clear-cut effect of sitaxentan compared to control in any of
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25 the five participants.
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28
29 It should be noted that the iontophoresis of ERAs was well tolerated both in animals
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31 and humans. No change in arterial blood pressure was observed and skin tolerance was
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33 excellent. Moreover, it did not induce any significant increase in aspartate transaminase or
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35 alanine transaminase in humans.
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39 In conclusion, this study shows that cathodal iontophoresis of sitaxentan but not
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41 bosentan partially reverses endothelin-induced skin vasoconstriction in rats, suggesting that
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43 sitaxentan diffuses into the dermis. We observed no effect of sitaxentan on basal skin
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45 microvascular tone in rats or humans.
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4
5 manuscript.
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10 **Conflicts of interest**

11
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Table 1. 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 (AUC₀₋₂₀) and during the whole recording (AUC₀₋₄₀).

Table 2. 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	%BL	19.3 ± 23.1	34.3* ± 15.5	8.21 ± 20	0.02
(n=8)	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	%BL	21.4 ± 26.2	26.4 ± 24.2	18.7 ± 24.4	0.84
(n=6)	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 (AUC₀₋₂₀) 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.

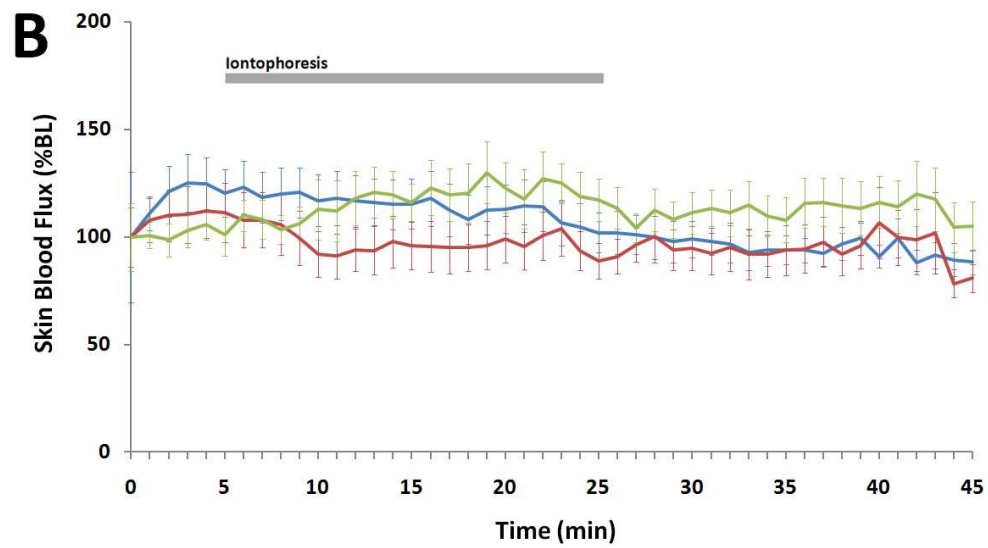
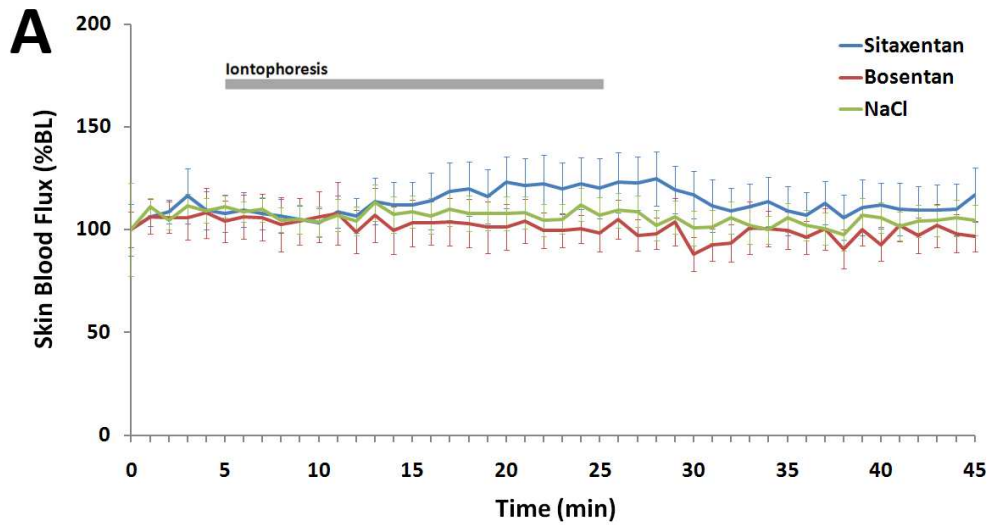
Figure legends

Figure 1: Effect of iontophoresis of bosentan 10^{-2} M, sitaxentan 10^{-2} M and NaCl 0.9 % on skin blood flux, expressed as mean (\pm SEM) percentage change from baseline (%BL), in the cathodal direction (Fig 1A) and the anodal direction (Fig 2B).

Figure 2: Typical tracing of the effect of ET-1 $0.5 \cdot 10^{-5}$ M on arterial pressure: the initial transient decrease of arterial pressure is followed by a sustained increase (Fig 2A). Mean (\pm SEM) arterial pressure during ET-1 perfusion in the cathodal (blue line) and anodal (red line) directions (Fig 2B).

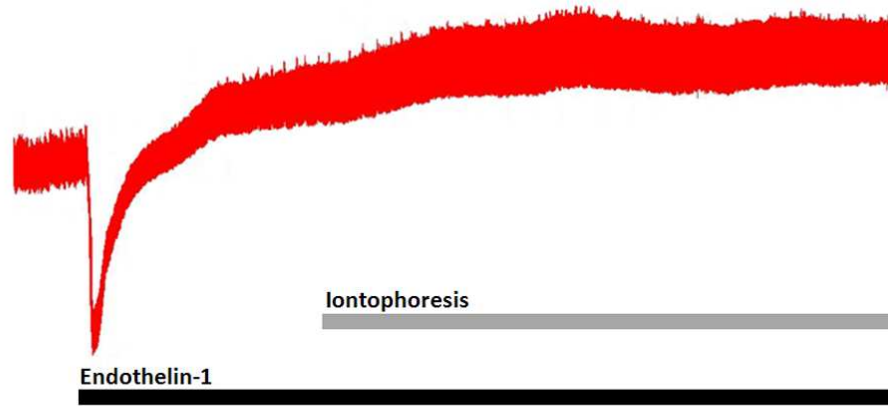
Figure 3: Effect of iontophoresis of bosentan 10^{-2} M, sitaxentan 10^{-2} M and NaCl 0.9 % on skin blood flux during infusion of ET-1 $0.5 \cdot 10^{-5}$ M, expressed as arbitrary perfusion units mean (mean \pm SEM), in the cathodal direction (Fig 3A) and anodal direction (Fig 3B).

Figure 4: Effect of iontophoresis of sitaxentan 10^{-2} M (red lines) and NaCl 0.9 % (blue lines) on skin blood flux, expressed as mean (\pm SEM) percentage change from baseline (%BL), in healthy volunteers' forearm skin (Fig 4A). Current intensity was set at 20 μ A (dashed lines) or 100 μ A (solid lines). Iontophoresis did not induce a significant change in mean arterial pressure (MAP) (Fig 4B).

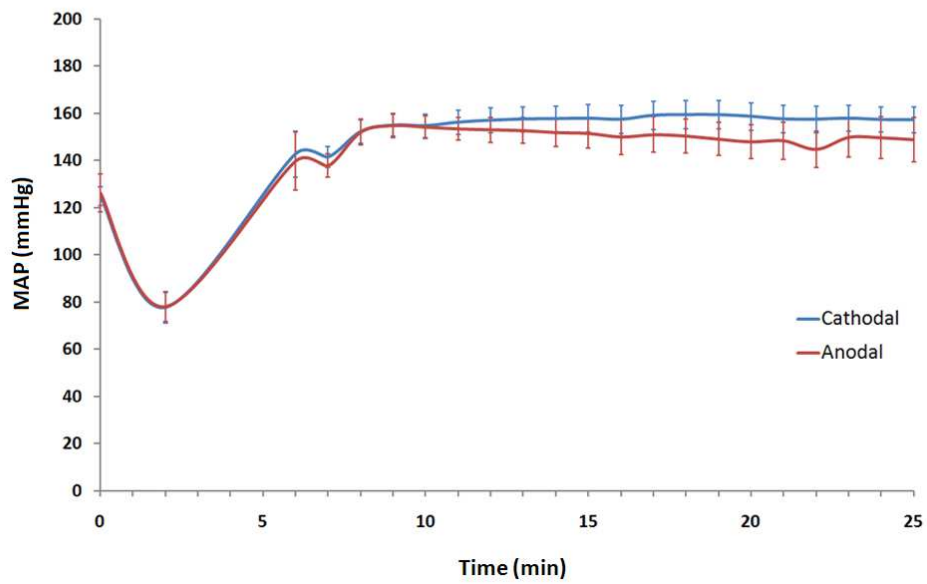


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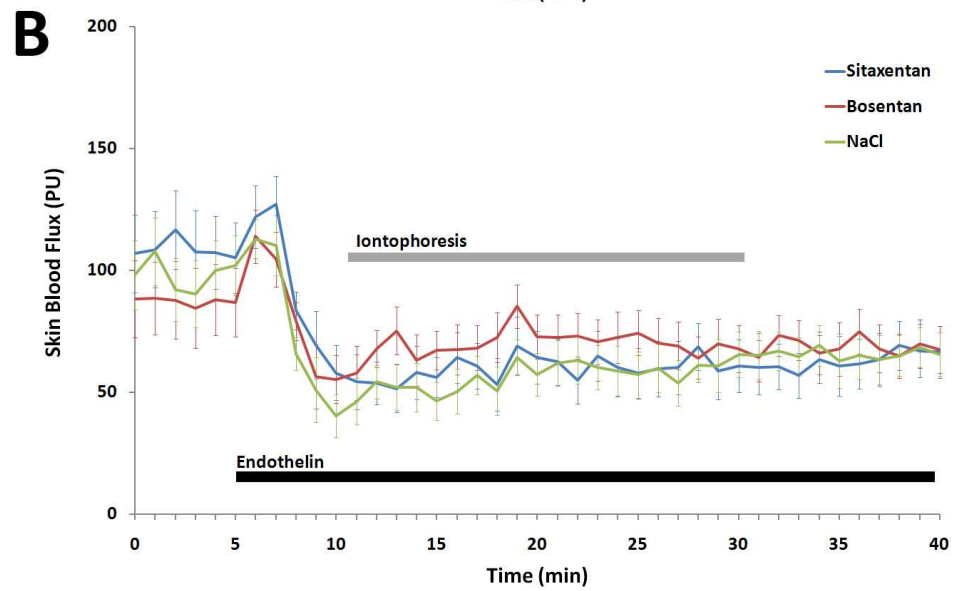
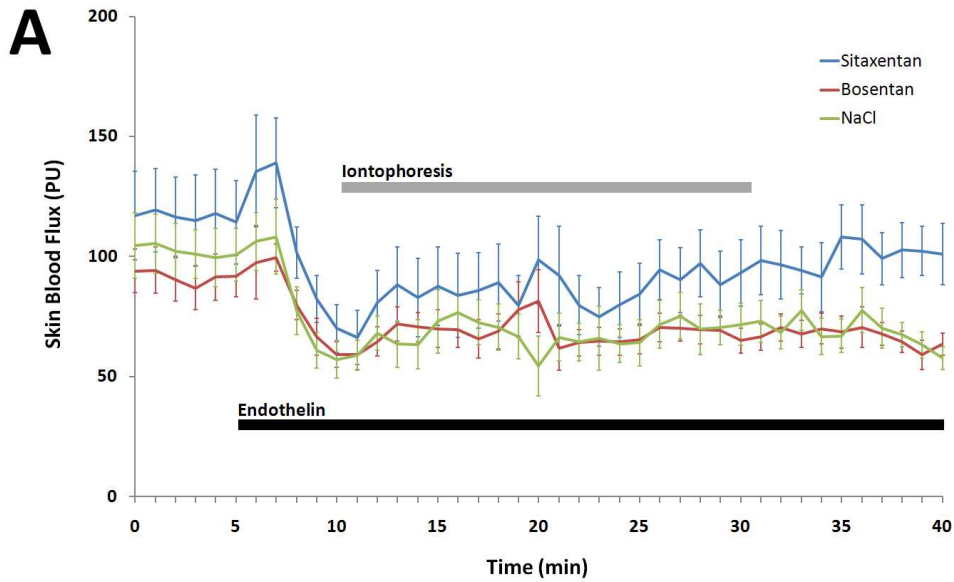


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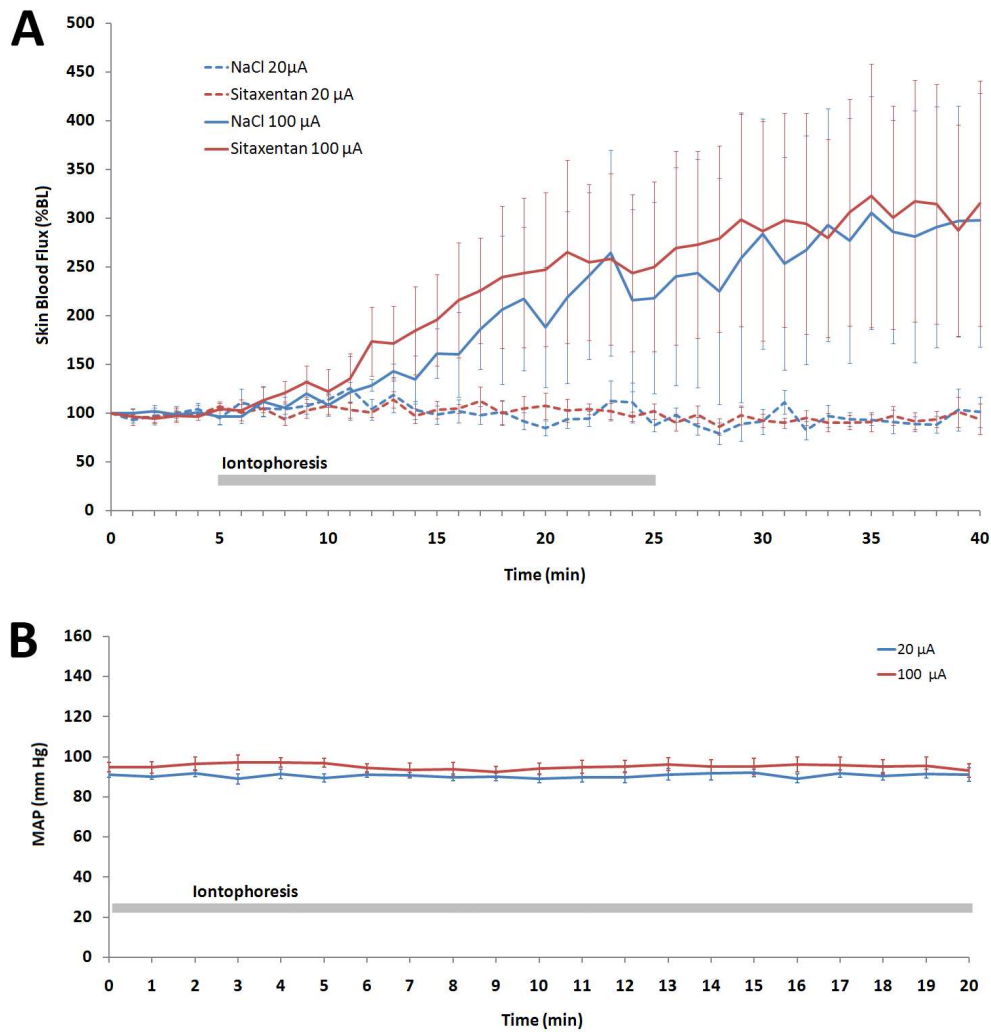


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Effect of iontophoresis of sitaxentan 10⁻² M (red lines) and NaCl 0.9 % (blue lines) on skin blood flux, expressed as mean (\pm SEM) percentage change from baseline (%BL), in healthy volunteers' forearm skin (Fig 4A). Current intensity was set at 20 μ A (dashed lines) or 100 μ A (solid lines).

Iontophoresis did not induce a significant change in mean arterial pressure (MAP) (Fig 4B).

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4 TROISIEME PARTIE : Iontophorèse thérapeutique de tréprostinil chez le volontaire sain : étude INFLUX-IT-VS

Les résultats encourageants chez le rat des analogues de la prostacycline nous ont permis d'élaborer des protocoles de iontophorèse chez le volontaire sain avec plusieurs molécules ayant des propriétés vasodilatatrices. Il a été décidé de retenir les deux molécules ayant démontré la meilleure dilatation vasculaire cutanée chez le rat : l'iloprost et le tréprostinil. Les schémas des iontophorèses et les concentrations utilisées ont été modifiés, le protocole ayant comme principal objectif de démontrer le concept de l'efficacité de la iontophorèse des analogues de la prostacycline, sans prendre de risque de toxicité, donc en testant des concentrations inférieures à celles testées chez l'animal. L'intensité des iontophorèses était de 20 μ A et prévoyait en cas d'inefficacité de pouvoir réaliser une iontophorèse de 100 μ A afin d'avoir la meilleure tolérance possible et d'éviter des effets secondaires à type de brûlure par exemple (peau humaine de l'avant-bras plus fine que celle du dos du rat où les iontophorèses étaient à 100 μ A). La concentration de tréprostinil a été diminuée afin de ne pas dépasser la dose totale maximale administrable chez l'homme car trois concentrations de tréprostinil étaient testées en simultanées (250 μ M, 25 μ M et 2,5 μ M au lieu de 6.4 mM, 0.64 mM et 0.064 mM chez le rat). La première partie du protocole chez 10 volontaires sains permettait de vérifier la faisabilité des iontophorèses. La iontophorèse d'iloprost a immédiatement été abandonnée du fait d'une toxicité locale cutanée. La iontophorèse de tréprostinil a pu être poursuivie avec dans une deuxième partie une modification des séances d'enregistrement LDI afin d'affiner les éléments cinétiques (scanner prolongé de LDI jusqu'à 24 heures après le début de la iontophorèse). Les résultats ont été concluants avec la démonstration d'une vasodilatation vasculaire cutanée significative et un effet « concentration » pour la iontophorèse de tréprostinil sur une durée d'environ 3 heures avec le schéma de iontophorèse de 20 μ A.

Article soumis en juillet 2011: *Cathodal iontophoresis of treprostinil induces a sustained increase in cutaneous blood flux in healthy volunteers.* S.Blaise, M.Roustit, M Hellmann, C.Millet, J.L. Cracowski.

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Cathodal iontophoresis of treprostinil induces a sustained increase in cutaneous blood flux in healthy volunteers.

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* M. Roustit and M. Hellmann contributed equally to this work

Running title: Cutaneous iontophoresis of treprostinil.

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Statement of conflicts of interest.

1
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4
5 being a consultant for Actelion. Dr Mathieu Roustit reports receiving grant support from
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7 Pfizer for another study
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10 **Keywords:** iontophoresis, prostaglandin I₂, prostaglandin analogue, treprostiril,
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For Peer Review

SUMMARY

Objective. Prostacyclin analogues are the most effective drugs to treat sclerodermic digital ulcers, but their systemic use is limited by their frequent side effects. We tested whether the prostacyclin analogues treprostinil and iloprost, delivered by cutaneous iontophoresis, induce sustained vasodilatation.

Methods. Twenty healthy volunteers received treprostinil by cathodal iontophoresis on the forearm. Skin blood flux was quantified using laser Doppler imaging.

Results. Treprostinil at 250 μM induced an increase in cutaneous vascular conductance $\text{AUC}_{80\text{min}}$ (31897 \pm 24390 %BL.min) compared to NaCl 0.9% ($P < 0.005$), treprostinil 2.5 μM ($P < 0.005$) and treprostinil 25 μM ($P < 0.005$). This was confirmed when flux was recorded for up to 10 h. Systemic and skin tolerance of treprostinil were good. Iloprost was stopped early because of local toxicity.

Conclusions. Treprostinil cathodal iontophoresis induces a sustained increase in cutaneous blood flow with good local tolerance. This could be investigated as a new local therapy for digital ulcers in scleroderma.

Clinicaltrials.gov (NCT01082484)

Introduction

Systemic sclerosis (SSc) is a rare disease affecting microcirculation and its treatment poses a difficult therapeutic challenge, particularly in the presence of cutaneous finger ulcers. Indeed, despite long-term treatment with calcium channel blockers patients are liable to serious handicap. Iloprost, a prostacyclin (PGI₂) analogue used intravenously, is the only drug currently approved for the treatment of existing digital ulcers¹. 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 ulcers². Ongoing studies are assessing the benefit of sildenafil, a type 5 phosphodiesterase inhibitor (PDE 5), on active ulcers^{3 4}. Epoprostenol (intravenous prostacyclin) and sildenafil are used as first or second line treatments in scleroderma patients with pulmonary arterial hypertension. In addition, treprostinil, a PGI₂ analogue, was approved by the Food Drug Administration for the treatment of pulmonary arterial hypertension by subcutaneous infusion in 2002 and by inhalation in 2009. However, the therapeutic effect of these drugs is counterbalanced by potentially serious systemic side-effects related to their potent vasodilatation properties.

Topical administration of these drugs may be a way of getting around the toxicity of systemic treatments. Iontophoresis, a non invasive controlled transdermal delivery system using a low-intensity electric current, could be an interesting alternative to ointments. Some authors suggest vasodilator iontophoresis as potentially interesting in the treatment of digital ulcerations in scleroderma^{5, 6}. However, a commonly used vasodilator such as sodium nitroprusside is not a good candidate given its short half-life and transient effect⁷. We recently showed that cathodal iontophoresis of iloprost and treprostinil increased cutaneous vascular conductance (CVC) on the hindquarter skin of rats⁸. Epoprostenol was also efficient but only at pH 12.

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3 Local administration of PGI₂ analogues by iontophoresis could therefore be of interest
4
5 for the treatment of digital ulcers. However, to our knowledge, iontophoresis of PGI₂
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7 analogues has never been tested in humans. The main objective of the present study was to
8
9 test whether treprostinil or iloprost iontophoresis increases skin blood flux on the forearm of
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11 healthy volunteers. Cutaneous microvascular effects of iloprost and treprostinil iontophoresis
12
13 were quantified using non-invasive laser Doppler Imaging (LDI) which can readily be used to
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15 assess skin blood flux^{9, 10}. We also assessed the cutaneous tolerance of treprostinil and
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20 iloprost.
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Patients and Methods

Study population

This was a proof of concept study, enrolling healthy subjects recruited through local newspaper advertisements. Inclusion criteria were an age of 18 years or older with no significant medical history. For female volunteers, pregnancy was excluded by urinary tests at the beginning of each visit. For all subjects, non-inclusion criteria included any allergies to local anesthetics, cigarette smoking or dermatologic disease on the arm or forearm. Grenoble Institutional Review Board (IRB n°6705) approval was obtained in November 2009 and each subject gave written informed consent before participation. The study was registered at clinicaltrials.gov (NCT01082484).

Drugs

The PGI₂ analogues we used were treprostinil (Bioprojet Pharma, Paris, France) 1 mg.mL⁻¹ solution (MW 390.5 g.mol⁻¹) and iloprost (Bayer Sante, Puteaux, France) 0.1mg.mL⁻¹ solution (MW 478.6 g.mol). We used isotonic sodium chloride (NaCl 0.9%) (Aguettant, Lyon, France) as a control.

Drugs were administered as commercially available solutions for human use and tested at 3 different concentrations: treprostinil solution (1 mg.ml⁻¹) was diluted with NaCl 0.9% to 0.1 mg.ml⁻¹ (250 μM), to 0.01 mg.ml⁻¹ (25 μM) and to 0.001 mg.ml⁻¹ (2.5 μM). Iloprost solution at 0.1mg.ml⁻¹ (200 μM) was used as such and diluted with NaCl 0.9% to 0.01 mg.ml⁻¹ (20 μM) and to 0.001 mg.ml⁻¹ (2 μM). The pH of iloprost and treprostinil solutions were 6 and 6.5, respectively, which is suitable for epidermal application. NaCl solution was chosen as control and diluant as it has been shown to induce less axon-reflex non-specific vasodilation in human skin than deionized water¹¹, as confirmed in our previous animal study⁸. All solutions were prepared extemporaneously.

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3 *Study design (Figure 1)*
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5 *Experiment 1: Dose dependent effect of the passive application and of 20 min cathodal*
6 *iontophoresis of treprostinil and iloprost, after one hour lidocaine/prilocaine pretreatment,*
7 *on cutaneous vascular conductance, recorded over 80 min*
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12 This was an open label pharmacology study enrolling ten healthy volunteers. Upon arrival at
13 the laboratory, subjects were placed in a temperature-controlled room (23+/-1°C) during 1
14 hour for acclimatization. They remained supine for the whole experiment. Blood pressure was
15 recorded continuously (Nexfin monitor, Bmeye B.V., Amsterdam, The Netherlands) on the
16 controlateral hand during skin blood flow measurements.
17

18 In the initial protocol, 6 consecutive visits were planned for each volunteer. Due to the early
19 discontinuation of the iloprost arm due to a skin adverse effect after the first enrollment, only
20 4 visits were performed. Therefore, each volunteer attended 4 consecutive visits at the
21 Clinical Research Center (Figure 1) separated by 7 days +/- 2. Visit 1 was the initial
22 enrollment visit. For the experimental visits the volunteers were fasted for at least 6 hours
23 before the visits and allowed to eat only after the end of the experiment. During visit 2, the
24 passive (i.e. without current) transdermal diffusion of treprostinil was studied at three
25 concentrations. During visit 3, iontophoresis of NaCl 0.9% and of treprostinil at the three
26 concentrations were realized. Visit 4 was a safety monitoring clinical visit. Skin photographs
27 were taken before the start of iontophoresis, immediately after iontophoresis and 7 days later.
28 The different visits are detailed below.
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53 *Passive cutaneous application of treprostinil and iloprost*
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55 We tested whether iloprost, treprostinil and NaCl 0.9% induce vasodilation through passive
56 diffusion. Before recording, the arm was immobilized with a vacuum cushion as previously
57 described¹². After the one hour acclimatization period at room temperature (23+/-1°C), four
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3 1.2 cm² circular iontophoresis chambers containing 360 µL were randomly placed on the
4
5 ventral side of the right forearm, more than 5 cm from the elbow and the prominence of the
6
7 wrist, avoiding visible veins. They were randomly filled with NaCl 0.9% (used as a control),
8
9 treprostinil at 250 µM, 25 µM or 2.5 µM, or NaCl 0.9%, iloprost 200 µM, 20 µM and 2 µM.
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12 Electrodes were not connected to the generator.
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15 Skin blood flux was assessed throughout the protocol using laser Doppler imaging (PeriIont
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17 System and PeriScan PIM 3 System, Perimed, Järfälla, Sweden), with the laser head placed
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19 20 cm above the skin. The resolution was set as medium (1 mm step length). Skin blood flux
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21 was recorded over 5 min before (baseline flux) and 80 min after the iontophoresis chambers
22
23 were positioned. At the end of the experiment, the chambers were removed and the skin was
24
25 heated locally at all locations, for 40 min at 43°C to attain maximal skin vasodilation, using a
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27 Perimed integrated heating probe (P457, Perimed, Järfälla, Sweden). Then maximum skin
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29 blood flux was recorded with LDI for 5 minutes. Preliminary experiments had shown that
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31 skin flux remained stable during the 5 min after probe removal.
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39 *Treprostinil iontophoresis protocol*

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41 Iontophoresis sites were chosen as described above and pre-treated with 1 g of
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43 lidocaine/prilocaine cream (5 g tubes containing 125 mg lidocaine and 125 mg prilocaine,
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45 Anesderm®, Pierre Fabre, Boulogne, France) in order to avoid axon reflex-induced
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47 hyperemia as previously described^{7, 13, 14}. An occlusive transparent dressing was placed over
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49 the cream to enhance cutaneous diffusion. Each anesthetized area was larger than the area of
50
51 the iontophoresis chambers.
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55 Before recording, the arm was immobilized with a vacuum cushion as described above
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57 (Figure 2). One hour after lidocaine/prilocaine application (corresponding to the
58
59 acclimatization period) the cream was removed with a cotton swab. Baseline flux was
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3 recorded by LDI for 5 minutes as described above. Skin blood flux was then recorded for 5
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5 additional minutes after the 4 chambers had been randomly filled with NaCl 0.9%, treprostinil
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7 250 μ M, 25 μ M and 2.5 μ M. Two passive electrodes (connected to the positive pole) were
8
9 placed approximately 15 cm from the iontophoresis chambers (connected to the negative
10
11 pole) (Figure 2). Cathodal iontophoresis was performed simultaneously at all sites for 20
12
13 minutes with a current intensity set at 20 μ A (Figure 1, experiment 1, visit 3). Skin blood flux
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15 was then recorded by LDI for 80 min. At the end of the experiment, the skin was heated at all
16
17 sites for 40 min at 43°C. Maximum skin blood flux was recorded for 5 minutes at the end of
18
19 the skin heating sequence by LDI.
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26 27 *Cutaneous resistance*

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29 During iontophoresis cutaneous resistance was determined with an amperometric biosensor
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31 unit (Multimeter system DVM1200, Velleman, Gavere, Belgium). For these measurements,
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33 one electrode was connected to the passive electrode and the other was sequentially connected
34
35 to each iontophoresis chamber. Voltage (expressed in volts) was then recorded and skin
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37 resistance calculated and expressed in Ω ¹⁵. Measurement started 5 min after the start of
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39 iontophoresis. This delay was chosen in pilot experiments to obtain stable voltage.
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46 ***Experiment 2: Dose dependent effect of 20 min cathodal iontophoresis of treprostinil and*** 47 ***iloprost without lidocaine/prilocaine pretreatment, on cutaneous vascular conductance,*** 48 ***recorded over 24 hours*** 49 50 51

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53 Since in experiment 1 treprostinil continued to have an effect on skin blood flux 80 min after
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55 the end of the iontophoresis, ten other healthy participants were recruited to test the duration
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57 of the effect.
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3 There were three visits separated by 7 days +/- 2. The first the initial enrollment visit, the
4
5 second the experimental visit and the last visit was a clinical safety visit (Figure 1).

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8 Experiment 2 proceeded in the same way as the first experiment (1 hour acclimatization in a
9
10 temperature-controlled room at 23+/-1°C) with the same material (Figure 2).

11
12 The treprostinil iontophoresis protocol was the same as above, except in four aspects: 1- there
13
14 was no anaesthesia prior to iontophoresis; 2- electrodes were removed immediately after the
15
16 end of iontophoresis; 3- immediately after the end of the 20 min iontophoresis treprostinil 250
17
18 µM was applied to a fifth site (Figure 2 top right) for 20 min; 4- skin blood flux was recorded
19
20 until 24 h after the end of iontophoresis as detailed below.
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23
24 After a 5 min baseline recording, skin blood flux was continuously recorded for 3 hours after
25
26 iontophoresis, then for 10 min every 30 min for 2 hours, and every hour for the next 5 hours
27
28 (Figure 1). After the initial 3 hour recording, subjects had to remain in the same acclimatized
29
30 room, but were free to move about between scans. Finally, skin blood flux was recorded again
31
32 24 h after iontophoresis. At the end of this protocol, the skin was heated as described above
33
34 and maximum skin blood flux was recorded over 5 minutes.
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38 Photographs of the skin were taken before the start of iontophoresis and at the safety visit.
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43 *Data analysis*

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45 Mean skin blood flux was computed over regions of interest covering the site of each
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47 iontophoresis electrode. Data were expressed as cutaneous vascular conductance (CVC),
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49 which is the flux in mV divided by the mean arterial pressure (MAP) in mmHg.
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51 Subsequently, we calculated the area under the curve of the CVC expressed as a percentage
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53 change from baseline, over 80 min ($AUC_{0-80min}$, protocol 1) and 10 hours (AUC_{0-10h} , protocol
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55 2).
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3 Continuous data were expressed as mean and standard deviation. Categorical data were
4 expressed as numerical values and percentage in parenthesis. Measurements from the same
5 subject were compared using a Friedman test followed by 2 x 2 Wilcoxon signed-rank tests
6 with each subject serving as his/her own control. P-values less than 0.05 corrected by
7 Bonferroni's correction were considered statistically significant. The data were analyzed
8 using SPSS 13.0.1 (SPSS Inc, Chicago, IL, USA). Ten subjects were enrolled in each sub-
9 study as a sample size of 9 in each group would have 80% power to detect a probability of 0.9
10 that the AUC with treprostinil 250 μM is higher than the AUC for NaCl 0.9% using a
11 Wilcoxon rank-sum test with a 0.05 two-sided significance level. (JD Elashoff; nQuery
12 Advisor[®] for Windows v 6.01, Statistical Solutions, MA, USA).

27 28 **Results**

29 30 *Protocol 1: Effect of the passive application and iontophoresis of treprostinil and iloprost* 31 *after local anesthesia on skin blood flux*

32 33 34 35 36 37 *Study population*

38 The demographic and clinical characteristics of the ten subjects were: 5 females and 5 males,
39 mean age 28.8 (8.91) years and body mass index 23.44 (2.87) kg/m^2 . Mean systolic/diastolic
40 arterial pressure at inclusion was 117.9 (10.6)/68.6 (10.2) mmHg.

41 42 43 44 45 46 47 48 49 *Passive cutaneous application of iloprost*

50 After the application of iloprost, without iontophoresis, at three different concentrations (200
51 μM , 20 μM and 2 μM) to the forearm of the first healthy volunteer, we observed a large
52 erythema in the probe area at each site of application, but predominantly at the site of the
53 highest concentration, immediately following the 20 min cutaneous application. The area
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3 affected persisted for 24 h, without any symptom of vesicles or bullae, and disappeared within
4
5 three days after application of moisturizing cream.
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8 The scientific committee concluded there to be localised skin toxicity, in addition to the
9
10 anticipated pharmacological cutaneous vasodilatation, and decided to stop the tests with
11
12 iloprost for the whole study.
13
14

15 16 17 *Passive cutaneous application of treprostinil*

18
19 Treprostinil at all three concentrations induced no significant increase in CVC compared to
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21 NaCl when applied without iontophoresis (Figure 3A). The $AUC_{0-80\text{min}}$ was 778 ± 1567
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23 %BL.min for NaCl 9%; 401 ± 2772 %BL.min for treprostinil $2.5 \mu\text{M}$; 2506 ± 3550
24
25 %BL.min for treprostinil at $25 \mu\text{M}$; 2479 ± 1567 %BL.min for treprostinil at $250 \mu\text{M}$
26
27 (Friedman test $P=0.24$) (Figure 3A). Data are shown as mean \pm standard deviation. The
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29 maximal increase in CVC induced by local heating for 40 min at 43°C did not differ between
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31 sites.
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38 39 *Effect of 20 min 20 μA cathodal iontophoresis of treprostinil at $250 \mu\text{M}$, $25 \mu\text{M}$ and $2.5 \mu\text{M}$* 40 41 *compared to NaCl 0.9%*

42
43 Treprostinil at $250 \mu\text{M}$ induced a significant increase in CVC compared with NaCl ($AUC_{0-80\text{min}}$
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45 were 31897 ± 24390 %BL.min and 133 ± 2407 %BL.min, respectively; $P<0.005$),
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47 compared with treprostinil at $25 \mu\text{M}$ (7084 ± 7704 %BL.min; $P<0.005$) and compared with
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49 treprostinil at $2.5 \mu\text{M}$ (970 ± 2706 %BL.min; $P<0.005$) (Figure 3B). Similarly, treprostinil at
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51 $25 \mu\text{M}$ significantly increased CVC compared to NaCl 0.9% ($P=0.007$). The maximal
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53 increase in CVC induced by local heating for 40 min at 43°C did not differ between
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55 treprostinil $250 \mu\text{M}$ (43.9 ± 10.9 mV/mmHg), treprostinil $25 \mu\text{M}$ (43.8 ± 12.4 mV/mmHg),
56
57 treprostinil $2.5 \mu\text{M}$ (45.7 ± 13.8 mV/mmHg) and NaCl 0.9% (47.7 ± 13.6 mV/mmHg),
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3 NS). Cutaneous resistance did not differ between sites for treprostinil 250 μ M (421 +/- 80
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5
6 K Ω), treprostinil 25 μ M (411 +/- 83 K Ω), treprostinil 2.5 μ M (409 +/- 83 K Ω) and NaCl
7
8 0.9% (420 +/- 86 K Ω , NS).
9

10 11 12 *Skin and systemic tolerance of the iontophoresis of treprostinil*

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15 No serious side-effect occurred. We observed no significant change in mean arterial pressure
16
17 after the iontophoresis of treprostinil (Figure 3C). Skin erythema localized to the probe area
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19 was observed as expected after removal of the heating probe, which disappeared in a few
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21 hours. No squamous, vesicular or bullous lesions were observed at the end of the experiment
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23 or at the safety visit.
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29 30 ***Protocol 2: Dose dependent effect of 20 min cathodal iontophoresis of treprostinil and*** 31 32 ***iloprost without lidocaine/prilocaine pretreatment on cutaneous vascular conductance,*** 33 34 ***recorded over 24 hours***

35 36 *Study population*

37
38 Five females and 5 males were included in this protocol. Their mean age was 22.7 (1.94)
39
40 years and body mass index was 23.31 (4.68) kg/m². The mean systolic/diastolic arterial
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42 pressure at inclusion was 126.8 (14.1)/72.4 (11.6) mmHg.
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49 50 *Effect of 20 min 20 μ A cathodal iontophoresis of treprostinil at 250 μ M, 25 μ M and 2.5 μ M* 51 52 *compared to NaCl 0.9% during 24 hours, expressed in AUC_{0-10h}*

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54 Treprostinil at 250 μ M induced a significant increase in cutaneous vascular conductance
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56 expressed as AUC_{0-10h} (1773 +/- 835 %BL.h) compared with NaCl 0.9% (285 +/- 288 %BL.h,
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58 P= 0.005), treprostinil at 25 μ M (488 +/- 287 %BL.h, P= 0.005), treprostinil at 2.5 μ M (273
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60 +/- 247 %BL.h, P= 0.005) and with the passive application of treprostinil 250 μ M (360 +/-

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3 593 %BL.h, $P= 0.005$) (Figure 4A). We observed an early flux peak in all groups except for
4 the passive application. The peak flux observed at 30 min was significantly higher for
5 the passive application. The peak flux observed at 30 min was significantly higher for
6 treprostiniil at 250 μM (703 \pm 246 %BL; $P= 0.005$ versus all groups except 25 μM). Peak
7 flux was 509 \pm 354 %BL for treprostiniil at 25 μM ; 320 \pm 192 %BL for treprostiniil at 2.5
8 μM , 335 \pm 274 %BL for NaCl 0.9% and 120 \pm 25 %BL for the passive application. At 24
9 hours, flux at all sites had returned to baseline. When NaCl 0.9% data were substracted from
10 treprostiniil 250 μM data, the peak was delayed by 1 hour (data not shown). The maximal
11 increase in CVC induced by local heating for 40 min at 43°C did not differ between sites:
12 treprostiniil 250 μM (39.94 \pm 9.8 mV/mmHg), treprostiniil 25 μM (40.17 \pm 7.3 mV/mmHg),
13 treprostiniil 2.5 μM (35.96 \pm 9.4 mV/mmHg) and NaCl 0.9% (35.42 \pm 10.5 mV/mmHg).
14
15 Cutaneous resistance did not differ between sites for treprostiniil 250 μM (384 \pm 133 K Ω),
16 treprostiniil 25 μM (383 \pm 151 K Ω), treprostiniil 2.5 μM (365 \pm 177 K Ω) and NaCl 0.9%
17 (368 \pm 154 K Ω , NS).
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37 *Skin and systemic tolerance of the iontophoresis of treprostiniil*

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39 No serious side effect occurred. We observed no significant drop in mean arterial pressure
40 after iontophoresis of treprostiniil (Figure 4B). Skin erythema persisted until 6 hours after
41 iontophoresis of treprostiniil at 250 μM and 25 μM . No squamous, vesicular or bullous lesions
42 were observed during the experiment or at the safety visit.
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Discussion

In this study we showed that the cathodal iontophoresis of 250 μM treprostinil induced a large and sustained increase in cutaneous blood flow on the forearm of healthy volunteers. The study confirms the proof-of-concept proposed by our previous animal study⁸. No effect of treprostinil was observed when applied without current, which confirms the validity of iontophoresis as a local route of administration for this drug.

This effect was observed using treprostinil concentrations at 250 μM and 25 μM compared to NaCl 0.9% in the first experiment which lasted for 80 min. Those results were confirmed by the second experiment which showed that cathodal iontophoresis of treprostinil at 250 μM induced a sustained increase in cutaneous blood flow in healthy volunteers compared to NaCl 0.9% and to treprostinil applied without iontophoresis of a similar order of magnitude as that in experiment 1.

Experiment 1 and 2 differ in several respects. In the first experiment, local anesthesia by pretreatment with lidocaine/prilocaine cream was used to avoid an axon reflex response during iontophoresis as already described¹³. As a consequence, iontophoresis of NaCl 0.9% did not increase CVC, suggesting that the effect of treprostinil was not due to an unspecific axon reflex. The second experiment was realized without lidocaine/prilocaine cream to assess the effect of treprostinil skin iontophoresis in standard conditions that could be used in a future clinical application. We observed an early peak at 30 min for all sites where iontophoresis was performed (including NaCl 0.9%) but not at the site where treprostinil 250 μM was applied without current. Moreover, we observed a trend towards a concentration-dependent effect on peak amplitude, suggesting that both the drug and unspecific an axon reflex are involved in this early response. In order to assess the kinetics of the pharmacological response, we subtracted the NaCl 0.9% recording from that for 250 μM treprostinil, and found the drug's peak to be delayed by 1 hour.

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3 Secondly, the second experiment showed that the CVC for the treprostinil 250 μ M site
4 returned to the control site values (NaCl 0.9%) after between 8 and 9 h. This delay was only 2
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6 hours for treprostinil at 25 μ M, which explains the significant difference with the control site
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8 observed in $AUC_{0-80\text{min}}$ (experiment 1) but not in $AUC_{0-10\text{h}}$ (experiment 2). We also observed
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10 a slight increase in CVC at all sites after 5 to 6 h that could be consecutive to a postprandial
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12 state.
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17 The third main difference was that in the second experiment the iontophoresis probes were
18 removed immediately after the end of the 20 min iontophoresis period, whereas in the first
19 they were removed after 80 min (20 min iontophoresis and 60 min recording. The reason for
20
21 the latter design was that we initially wanted to reproduce the data we recently observed with
22
23 rats, with minimal change in the area covered by the probe and to avoid artefacts following
24
25 probe removal. The second experiment shows that 20 min treprostinil iontophoresis followed
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27 by immediate removal of the probe is sufficient to induce a sustained increase in CVC.
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33 One potential issue for this study was that we used NaCl 0.9% rather than treprostinil buffer
34 as a control. We had previously shown that pure treprostinil buffer did not alter skin blood
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36 flow in animals, while treprostinil did ⁸. Moreover, we used treprostinil solution diluted in
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38 NaCl 0.9% even at the highest concentration, and therefore the choice of NaCl as a control
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40 seemed relevant. Lastly, we were unable to obtain Good Manufacturing Practice labelled
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42 treprostinil placebo from the manufacturer, and did not have the possibility to locally
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44 reconstitute treprostinil buffer for human use, as we had done for rats that did not need such
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46 stringent requirements ⁸.
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52 We chose to express data as a percentage of baseline rather than as a percentage of maximal
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54 cutaneous vascular conductance given the fact that in our future protocol studying wider areas
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56 and different skin territories we will not be able to use local heaters and therefore measure the
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3 maximal CVC. In addition, initial rat data were expressed as such ⁸. Therefore, this way of
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6 expressing data will enable us to compare these experiments.

7
8 The effect of treprostinil was more pronounced in humans than in rats, despite lower drug
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10 concentrations and a lower current (20 instead of 100 μ A). This is consistent with the similar
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12 trend towards a greater effect in humans than in rats that we observed with the iontophoresis
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14 of sodium nitroprusside ^{7, 8}. These differences may be explained by differences in skin
15
16 structure. In the human study, the addition of treprostinil did not alter skin resistance in either
17
18 of the experiments, as previously observed in rats ⁸.

19
20
21 A good candidate for therapeutic iontophoresis for human use would be an ionized drug
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23 which would induce a sustained increase in cutaneous blood flow at the site of transdermal
24
25 delivery without cutaneous toxicity. The passive application of iloprost induced a sustained
26
27 large erythema in the first healthy volunteer which led us to stop the experiment for safety
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29 reasons. The treprostinil concentration used was from 1/10th to 1/50th of that used for
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31 subcutaneous treatment in pulmonary arterial hypertension. Apart from the expected transient
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33 erythemas, this probably explains why we observed no local side-effect such as the localized
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35 pain often described following subcutaneous infusion. Moderate erythema has been described
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37 as a possible side effect of iontophoresis ¹⁶ with tingling-like sensations. We observed no
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39 systemic effects, in particular in mean arterial blood pressure that was monitored
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41 continuously. A previous report showed that sodium nitroprusside and acetylcholine lead to
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43 increased digital flux in patients with SSc ⁶. This suggests that iontophoresis could be tested
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45 as a new treatment for digital ulcers in scleroderma, despite dermal thickening and fibrosis.
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48 Our data suggest that treprostinil is a good candidate drug given the pharmacodynamic profile
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51 we observed.
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3 In conclusion, cutaneous cathodal iontophoresis of 250 μ M treprostinil induced a large and
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5 sustained increase in cutaneous blood flow in healthy volunteers, without systemic or topical
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7 side-effects. The kinetics of the increased CVC following iontophoresis of treprostinil would
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9 be compatible with intermittent iontophoresis of the drug. Therefore, topical delivery of
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11 treprostinil via iontophoresis could be considered as an alternative method of administration
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13 to increase the drug concentration locally avoiding its major systemic adverse effects.
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15 Treprostinil cathodal iontophoresis should be further investigated as a new local therapy for
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17 digital ulcers in patients with scleroderma.
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Legends for illustrations

Figure 1: Study scheme for the two experiments on cathodal iontophoresis of treprostinil on the forearm of a healthy volunteer (n= 10 for each experiment). LDI: laser doppler imaging. Iontophoresis: iontophoresis of treprostinil. The blue area corresponds to the time when LDI was recorded on the forearm.

Figure 2: Experimental setup: four iontophoresis chambers (A) connected to power supplies (B) were placed on the ventral face of the right forearm of a healthy volunteer. Laser Doppler imaging (C) was performed over the area including all iontophoresis chambers. Blood pressure was continuously recorded on the controlateral hand using photoplethysmography (Nexfin monitor, D). Right top: detailed overview of the forearm. In experiment 1 and 2, the 4 iontophoresis probes were placed randomly. In experiment 2, a fifth probe was placed after the end of the iontophoresis and filled with treprostinil 250 μM as a control for passive application. The reason for the fifth probe being added after iontophoresis was to ensure that no electric current was delivered while recording passive diffusion.

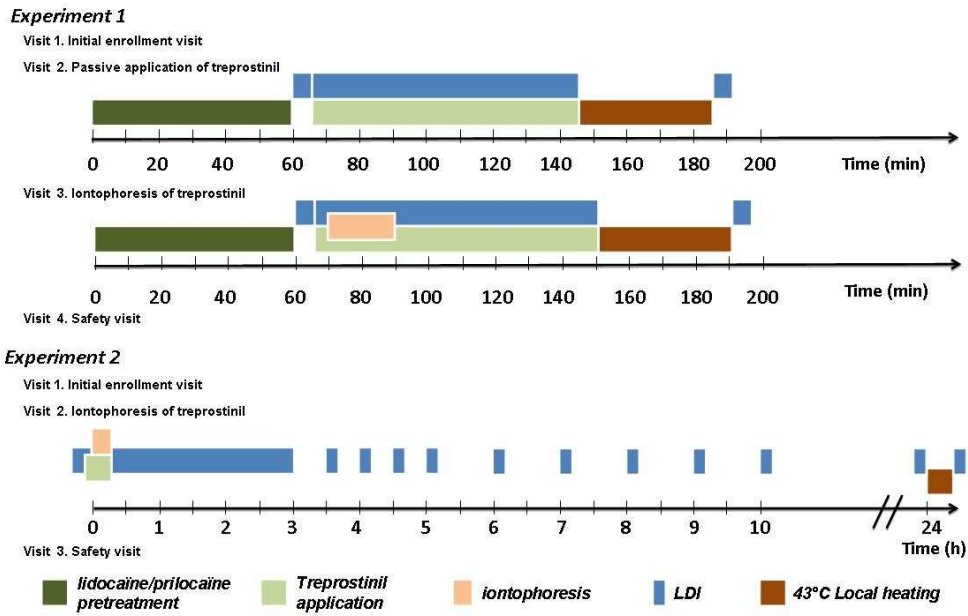
Figure 3: Effect of the application to the forearm without (passive) (A) or with cathodal iontophoresis (20 min 100 μA) (B) of treprostinil at 250 μM , 25 μM and 2.5 μM , versus NaCl 0.9%, on cutaneous vascular conductance (CVC) expressed as a percentage of baseline, recorded over 80 min. Treprostinil was applied one hour after lidocaine/prilocaine pretreatment. Continuous mean arterial pressure (MAP) recording (C), expressed in mmHg, during and after forearm skin iontophoresis of treprostinil at 250 μM , 25 μM and 2.5 μM , versus NaCl 0.9%.

Figure 4: Effect of iontophoresis (20 min 100 μA) on the forearm of treprostinil at 250 μM , 25 μM and 2.5 μM , versus NaCl 0.9%, on cutaneous vascular conductance, expressed as a percentage of baseline recorded up to 24 hours (A). Continuous mean arterial pressure (MAP)

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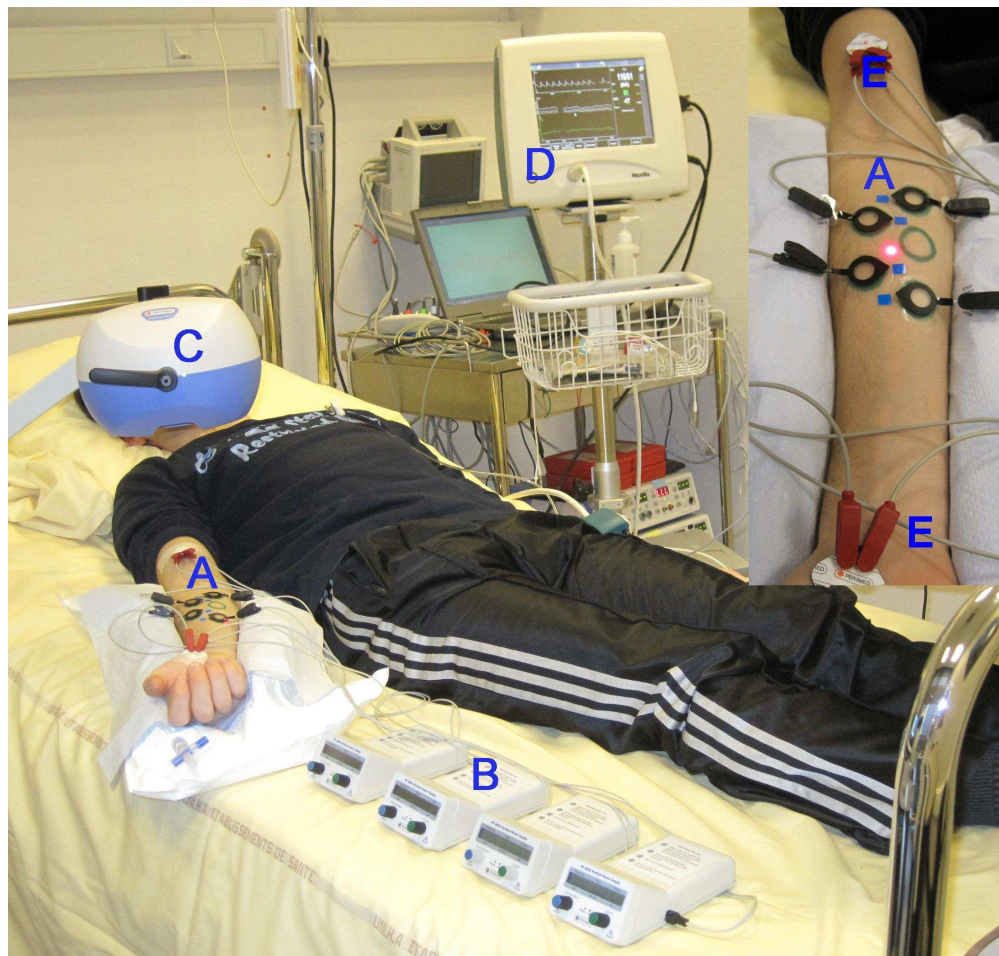
16 **Statement of conflicts of interest.**
17

18 Pr Jean-Luc Cracowski reports receiving grant support from Pfizer for another study and
19 being a consultant for Actelion. Dr Mathieu Roustit reports receiving grant support from
20 Pfizer for another study.
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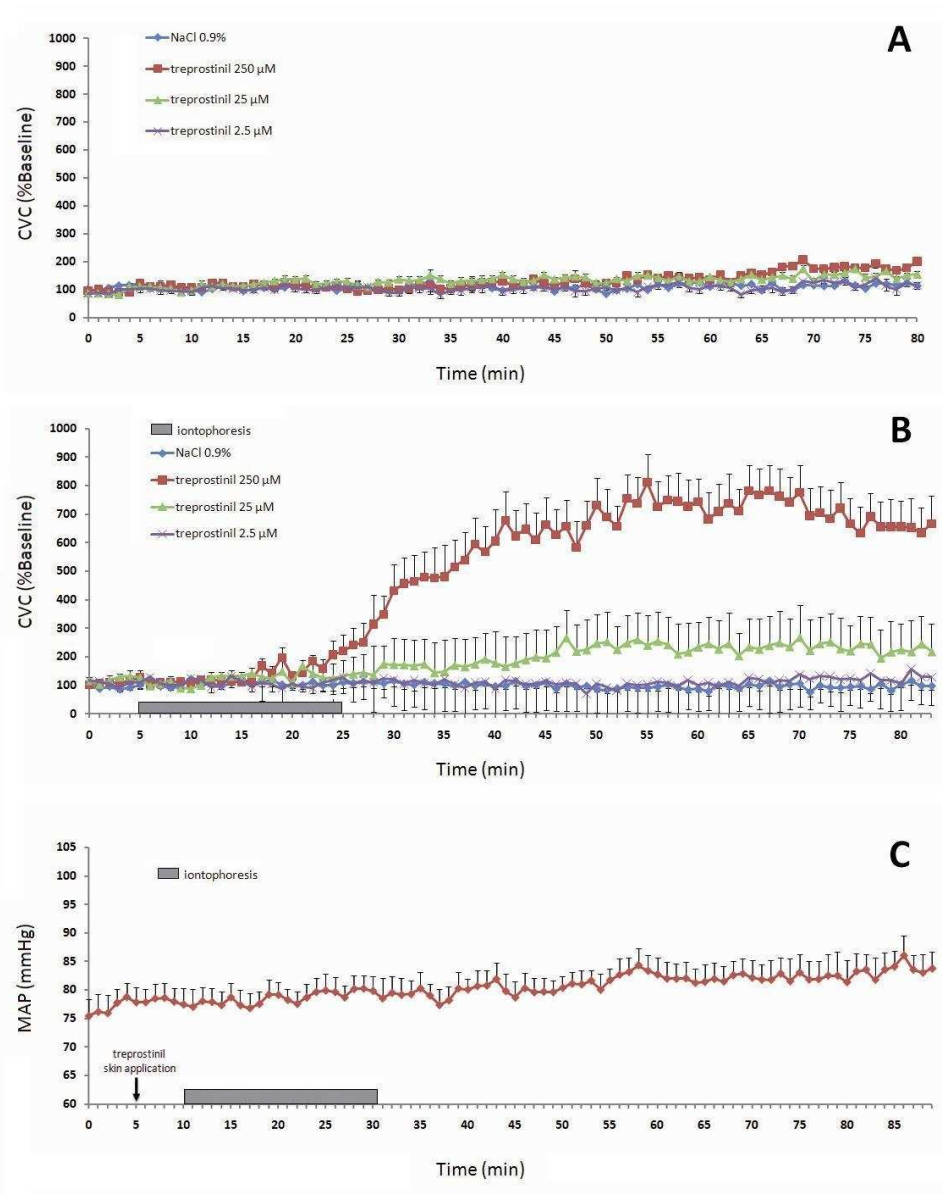
Study scheme for the two experiments on cathodal iontophoresis of treprostinil on the forearm of a healthy volunteer (n= 10 for each experiment). LDI: laser doppler imaging. Iontophoresis: iontophoresis of treprostinil. The blue area corresponds to the time when LDI was recorded on the forearm.

300x190mm (96 x 96 DPI)



Experimental setup: four iontophoresis chambers (A) connected to power supplies (B) were placed on the ventral face of the right forearm of a healthy volunteer. Laser Doppler imaging (C) was performed over the area including all iontophoresis chambers. Blood pressure was continuously recorded on the contralateral hand using photoplethysmography (Nexfin monitor, D). Right top: detailed overview of the forearm. In experiment 1 and 2, the 4 iontophoresis probes were placed randomly. In experiment 2, a fifth probe was placed after the end of the iontophoresis and filled with treprostinil 250 μM as a control for passive application. The reason for the fifth probe being added after iontophoresis was to ensure that no electric current was delivered while recording passive diffusion.

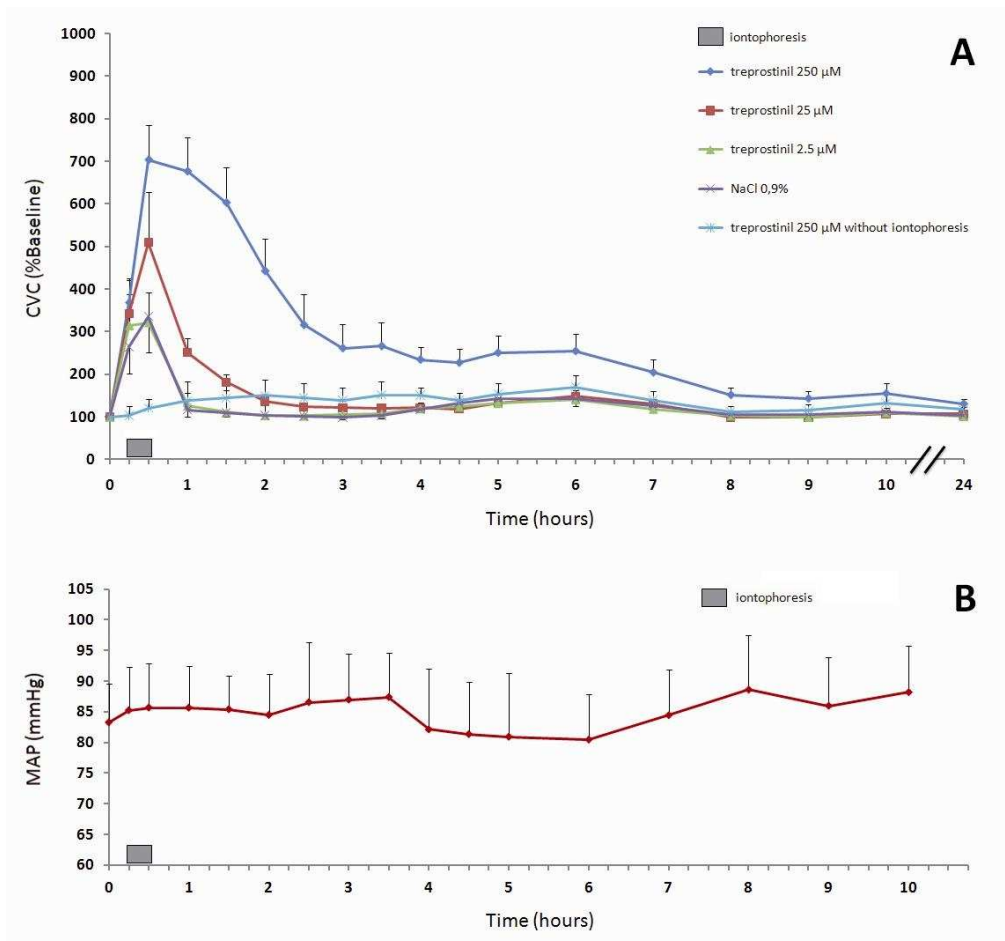
313x298mm (180 x 180 DPI)



Effect of the application to the forearm without (passive) (A) or with cathodal iontophoresis (20 min 100 μ A) (B) of treprostinil at 250 μ M, 25 μ M and 2.5 μ M, versus NaCl 0.9%, on cutaneous vascular conductance (CVC) expressed as a percentage of baseline, recorded over 80 min. Treprostinil was applied one hour after lidocaine/prilocaine pretreatment. Continuous mean arterial pressure (MAP) recording (C), expressed in mmHg, during and after forearm skin iontophoresis of treprostinil at 250 μ M, 25 μ M and 2.5 μ M, versus NaCl 0.9%.

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Effect of iontophoresis (20 min 100 μA) on the forearm of treprostinil at 250 μM, 25 μM and 2.5 μM, versus NaCl 0.9%, on cutaneous vascular conductance, expressed as a percentage of baseline recorded up to 24 hours (A). Continuous mean arterial pressure (MAP) recording (B), expressed in mmHg, during and after forearm skin iontophoresis of treprostinil at 250 μM, 25 μM and 2.5 μM, versus NaCl 0.9%.

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5 QUATRIEME PARTIE : Autres travaux sur le sujet

5.1 Développement de la reproductibilité des méthodes d'explorations en microcirculation

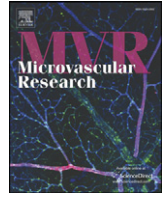
Le laboratoire possède plusieurs appareils d'exploration non invasive ou invasive pour étudier la microcirculation (cf chapitre 1.3). L'évolution rapide technologique notamment en matière d'acquisition des images ne doit pas faire oublier le travail nécessaire à la validation des outils, notamment en termes de reproductibilité des techniques. Le laboratoire a suivi l'évolution des technologies avec l'acquisition à son début d'un LDF, puis d'un LDI puis d'un LSCI.

Un premier travail a été effectué pour évaluer la reproductibilité à une semaine d'intervalle de la PORH et de l'hyperhémie post chauffage par le LDF selon différents facteurs : la reproductibilité spatiale (en évaluant les tests microcirculatoires sur différents sites cutanés de l'avant-bras et de la pulpe), la température (21°C et 27°C) et le stress (Stroop test). L'étude a inclus 14 volontaires sains. La PORH était reproductible sur le doigt en valeur absolue de CVC (coefficient de variation (CV)=25%; coefficient de corrélation intra-classe (ICC)=0.56) ou en % de CVC (CV=24%; ICC=0.60). Nous concluons à une reproductibilité médiocre au niveau de l'avant-bras. Les résultats sont identiques avec un préchauffage à 33°C avec une reproductibilité au niveau du doigt en CVC (CV=17%; ICC=0.81) et une reproductibilité médiocre au niveau de l'avant-bras. Une température de la pièce élevée à 27°C altérerait les résultats de PORH et de chauffage au niveau du doigt mais pas au niveau de l'avant-bras. L'étude concluait à une bonne reproductibilité du LDF pour la PORH et le chauffage au niveau du doigt lors que l'expression des résultats en valeur absolue de CVC ou en % de CVC. La mauvaise reproductibilité au niveau de l'avant-bras pourrait être expliquée par la variabilité spatiale et l'hypothèse d'une variabilité de la densité capillaire.

Article publié: [Reproducibility and methodological issues of skin post-occlusive and thermal hyperemia assessed by single-point laser Doppler flowmetry.](#) Roustit M, Blaise S, Millet C, Cracowski JL. *Microvasc Res.* 2010 Mar;79(2):102-8. Epub 2010 Jan 11.

Un deuxième travail a été effectué pour comparer la reproductibilité du LDF et du LDI sur l'évaluation de la PORH et la comparaison du LDF, LDI et LSCI dans l'hyperhémie post chauffage local. Une étude a été réalisée avec 38 volontaires sains. La reproductibilité du pic de la PORH était meilleure avec le LSCI par rapport au LDF (CV=8%; ICC=0.76 pour le LSCI et CV=30%; ICC=0.54 pour le LDF). La reproductibilité inter-sujet du chauffage était meilleure avec le LSCI et le LDI qu'avec le LDF (CV=15%, ICC=0.66; CV=17%, ICC=0.51 and CV=42%, ICC=0.28 respectivement). Il existait une corrélation entre les mesures du pic CVC de PORH entre le LDF et le LSCI (R=0.54; p=0.001). Ces résultats vont dans le sens d'une bonne reproductibilité du LSCI à la fois pour l'étude de la PORH et de l'hyperhémie post chauffage et une bonne corrélation en un point entre le LDF et le LSCI pour l'étude de la PORH.

Article publié: [Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity.](#) Roustit M, Millet C, Blaise S, Dufournet B, Cracowski JL. *Microvasc Res.* 2010 Mar;79(2):102-8. Epub 2010 Jan 11.



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 (%CVC₄₄). 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 %CVC₄₄ (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 %CVC₄₄. 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.

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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 cyclooxygenases 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 (%CVC₄₄), 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).

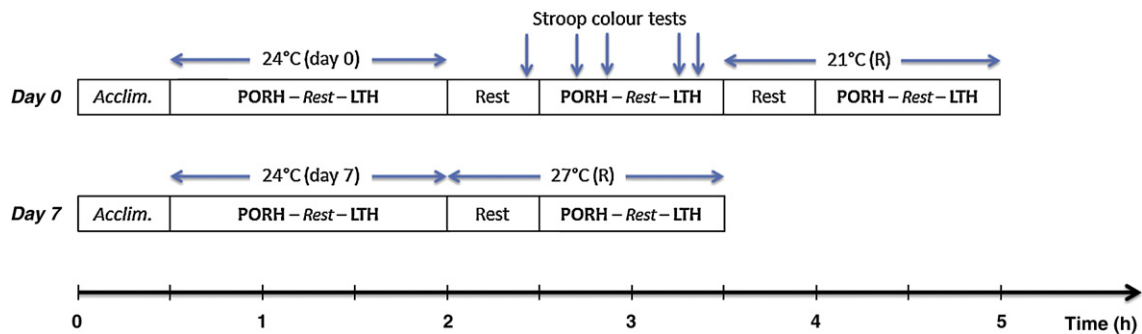


Fig. 1. The study design included two visits, 7 days apart. One week reproducibility of post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) was assessed between two measurements recorded at the same hour at 24 °C. The Stroop color test was performed on day 0 at 24 °C. Afterward,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

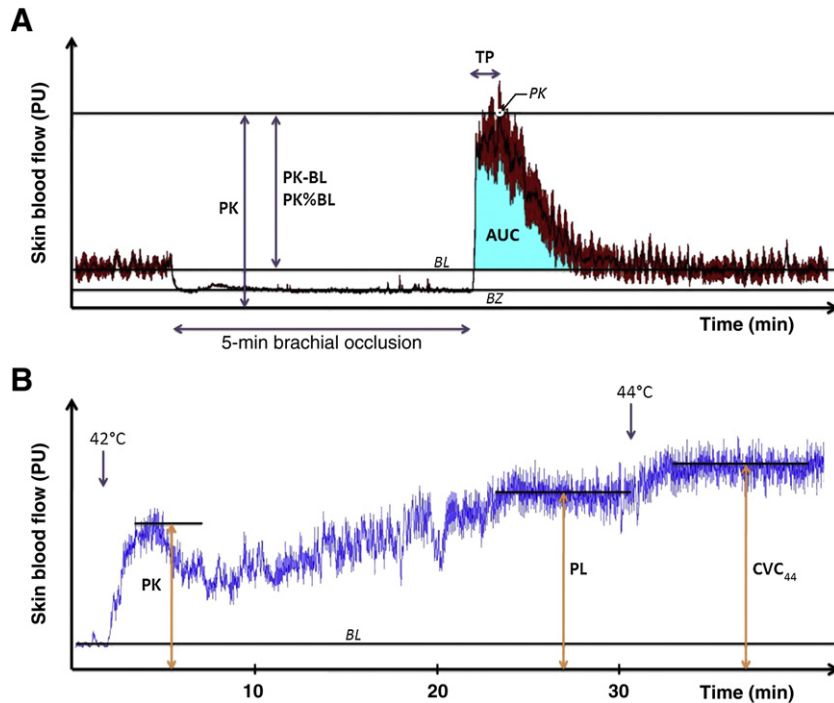


Fig. 2. Examples of post-occlusive reactive hyperemia (PORH) and local thermal hyperemia (LTH) performed on the forearm. (A) PORH data are expressed as peak raw value (PK), peak minus baseline (PK-BL), the percentage increase between peak and baseline (PK%BL), the area under the curve (AUC) and the percentage of vasodilation to 44 °C local heating (B, CVC₄₄). 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 (CVC₄₄).

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 CVC₄₄. 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 %CVC₄₄ (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 (%CVC₄₄). 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 (%CVC₄₄) 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 CVC₄₄. Spatial reproducibility of LTH was good only when expressed as %CVC₄₄ 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 (%CVC₄₄). 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 (%CVC₄₄) 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 CVC₄₄ 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 CVC₄₄ (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 %CVC₄₄ (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 %CVC₄₄. 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 %CVC₄₄ 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 %CVC₄₄. 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-dependent 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 (Torggrimson 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 %CVC₄₄, 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 CVC₄₄ 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.

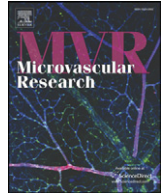
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Regular Article

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.

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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 cm². 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/cm²). The image acquisition rate was 8 s⁻¹ and frames were 81 cm².

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 cm² 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

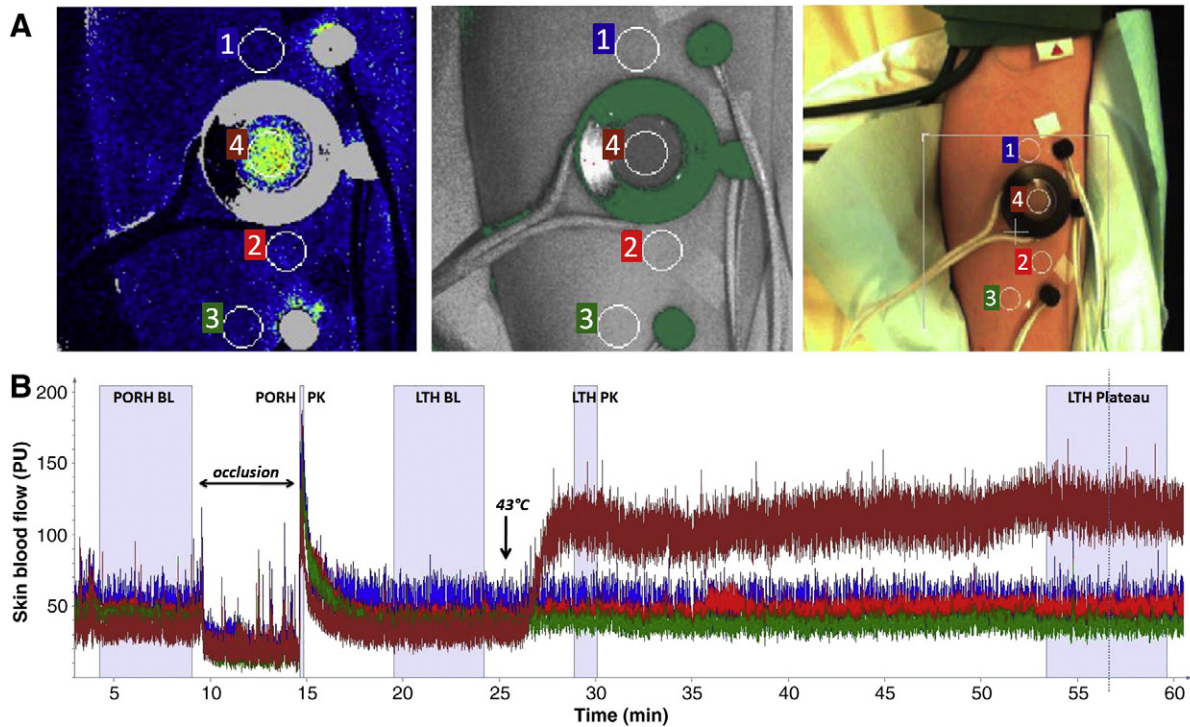


Fig. 1. (A) Laser speckle contrast imaging (LSCI) estimated perfusion (left; from low perfusion in black to high perfusion in red), intensity (middle) and picture (right) on the forearm. Regions of interests (ROIs) were adjacent to the LDF probes (numbered 1–3) and within the SHP3 heating probe (4). (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

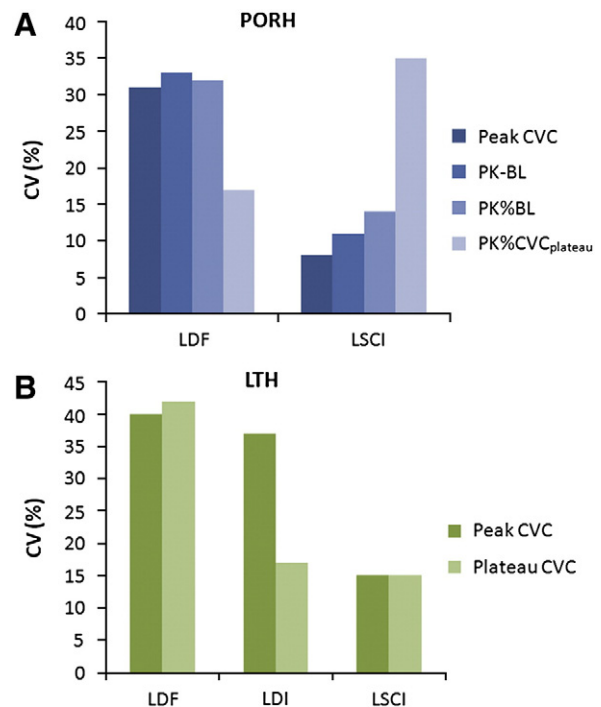


Fig. 2. (A) Inter-day reproducibility of post-occlusive reactive hyperemia (PORH) assessed with laser Doppler flowmetry (LDF) and laser speckle contrast imaging (LSCI). Data are expressed as within subject coefficients of variation (CV, in %) of peak cutaneous vascular conductance (Peak CVC, in mm Hg), 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.

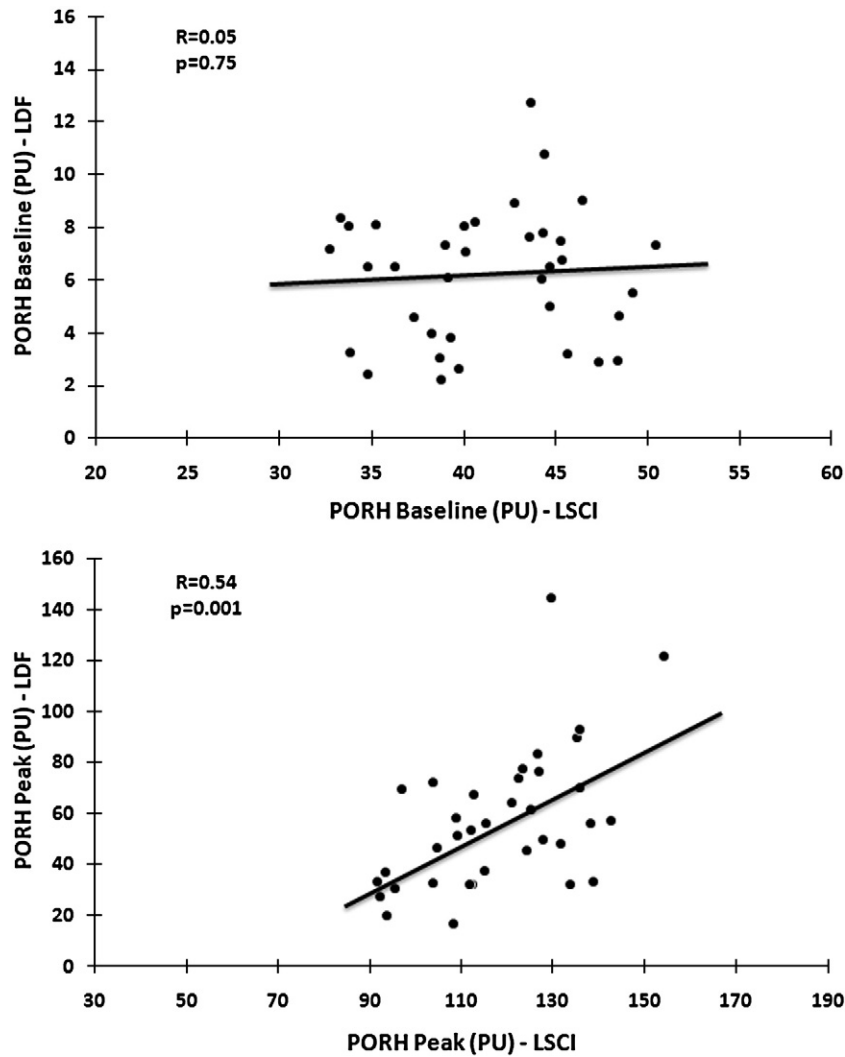


Fig. 3. Correlation between simultaneous assessment of post-occlusive reactive hyperemia (PORH) baseline (up) and peak (down), with laser Doppler flowmetry (LDF) and laser speckle contrast imaging (LSCI) (Pearson correlation test). Data are expressed as blood flux in perfusion units (PU).

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 mm² 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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5.2 Etudes en physiologie de la microcirculation

La volonté de développer des outils thérapeutiques va de paire avec une meilleure connaissance de la physiopathologie. Le laboratoire réalise ainsi des études en physiologie microcirculatoire chez le volontaire sain, les patients avec RP et les patients ScS. Une étude a été réalisée en évaluant le flux microcirculatoire chez 6 patients ScS et 6 RP en réponse à une iontophorèse de SNP en comparant les réponses au niveau de l'avant-bras et du bras avec le LDF. Les iontophorèses de SNP et de NaCl ont été réalisées au niveau de l'avant-bras et de la pulpe du doigt avec et sans lidocaine/prilocaine. Les iontophorèses de SNP induisent une dilatation microcirculation au niveau de l'avant-bras dans les deux groupes, mais pas au niveau de la pulpe du doigt dans le groupe ScS (excepté un patient ScS). L'équipe concluait donc à l'utilisation d'autres moyens d'exploration que la iontophorèse de SNP pour l'évaluation de la fonction microcirculatoire acrale non endothélium dépendante chez le patient ScS.

Article publié: [Sodium nitroprusside iontophoresis on the finger pad does not consistently increase skin blood flow in healthy controls and patients with systemic sclerosis.](#) Roustit M, Blaise S, Cracowski JL. *Microvasc Res.* 2009 May;77(3):260-4. Epub 2009 Mar 6

La dysfonction vasculaire endothéliale peut également être explorée par des marqueurs biologiques. L'intrication entre la dysfonction vasculaire endothéliale et des marqueurs liés également à la dysfonction fibrosante est une piste à étudier. Le laboratoire a participé à une étude avec des patients ScS avec l'évaluation de deux marqueurs dont on savait qu'ils étaient augmentés dans la ScS : un marqueur de dysfonction vasculaire (un inhibiteur endogène de l'acide nitrique synthétase, l'ADMA (acide diméthylarginine asymétrique plasmatique) dosé par spectrochromatographie liquidienne de masse) et un marqueur du remodelage matriciel, un inhibiteur de la métalloprotéase1 (TIMP-1) dosé par la technique Elisa. Les résultats retrouvaient des taux les plus élevés des deux marqueurs dans le groupe ScS avec surtout une corrélation entre l'ADMA et le TIMP-1 dans le groupe ScS ($r = 0.34$, $p = 0.035$). Ces résultats alimentent l'hypothèse du lien entre le remodelage matriciel et la dysfonction endothéliale dans la physiopathologie de la ScS. (Blaise *et al.*, 2009)

Article publié: [Correlation of biomarkers of endothelium dysfunction and matrix remodeling in patients with systemic sclerosis.](#) Blaise S, Maas R, Trocme C, Kom GD, Roustit M, Carpentier PH, Cracowski JL. *J Rheumatol.* 2009 May;36(5):984-8. Epub 2009 Mar 30

Le phénomène de Raynaud (RP) est une des manifestations vasculaires dans la ScS. La structure morphologique des capillaires dans le RP primaire semble être normale, alors que certains suggèrent déjà une altération fonctionnelle microcirculatoire. Une étude a été menée comparant la réponse au froid (sonde prototype développée dans le service) induisant une température locale à 15°C ou 24°C pendant 30 minutes au niveau de l'avant-bras et du doigt chez deux groupes de sujets (21 RP et 20 volontaires sains). L'évaluation de la microcirculation était faite par le LDF après un traitement par lidocaine/prilocaine. Les résultats étaient exprimés en CVC, AUC 0-30 minutes et CVC % BL. Les CVC de la face dorsale du doigt étaient plus basses dans le groupe RP par rapport au groupe contrôle à 5°C et à 24°C alors qu'aucune différence n'était mise en évidence au niveau de la pulpe et de l'avant-bras. L'anesthésie locale augmentait la CVC dans le groupe RP mais pas dans le

groupe contrôle. La vasoconstriction cutanée augmentée lors de la réponse locale au froid au niveau du doigt chez les RP primaires pourrait dépendre partiellement de l'anomalie à la réponse nerveuse sensitive. (Roustit *et al.*)

Article publié: *Impaired transient vasodilation and increased vasoconstriction to digital local cooling in primary Raynaud's phenomenon.* Roustit M, Blaise S, Millet C, Cracowski JL. Am J Physiol Heart Circ Physiol. 2011 May 13.



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Sodium nitroprusside iontophoresis on the finger pad does not consistently increase skin blood flow in healthy controls and patients with systemic sclerosis[☆]M. Roustit, S. Blaise, J.L. Cracowski^{*}

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ABSTRACT

Objective: Sodium nitroprusside (SNP) iontophoresis is a commonly used technique to assess non endothelium-dependent skin microvascular function in the forearm. However, the lack of data on the finger pad is a limitation when studying diseases affecting the digits (e.g. systemic sclerosis, SSc). We thus aimed to validate this technique in the finger pad compared to the forearm in SSc patients and healthy controls.

Methods: Six SSc patients and six controls were recruited. SNP and NaCl iontophoresis were performed on the finger pad and the forearm, with and without lidocaine/prilocaine. Cutaneous blood flow was simultaneously monitored using laser Doppler flowmetry.

Results: In all subjects, iontophoresis of SNP induced hyperemia in the forearm, which was not affected by pretreatment with lidocaine/prilocaine. In contrast, no increase in cutaneous vascular conductance was observed in the finger pad in any subject (apart from one patient with SSc).

Conclusions: The iontophoresis of SNP leads to a consistent, non axon reflex-dependent, increase in cutaneous vascular conductance in the forearm, both in patients with SSc and in healthy controls. On the finger pad however, such hyperemia was not consistent. As a consequence, other tools should be considered to assess non endothelium-dependent skin microvascular function in the finger pad.

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Introduction

Microvascular function can routinely be studied in human skin using non invasive laser Doppler flowmetry (Cracowski et al., 2006). Similarly to the flow mediated dilation of the brachial artery, discrimination between the endothelium and the non endothelium-dependent cutaneous microvascular responses remains an issue. Post-occlusive reactive hyperemia (PORH), thermal hyperemia and acetylcholine iontophoresis are commonly used as functional markers of endothelial microvascular function (Cracowski et al., 2006). On the other hand, assessment of non endothelium-dependent microvascular function mainly relies on sodium nitroprusside (SNP) iontophoresis. Indeed, SNP is a donor of NO, which leads to an activation of guanylate cyclase and an increased production of cyclic guanosine monophosphate (cGMP) in smooth muscle cells. The resulting vasodilation is therefore not dependent on the endothelial production of NO.

Most data available on SNP iontophoresis concerns the skin of the forearm. However, diseases such as Raynaud's disease and systemic sclerosis (SSc) preferentially affect the digits (Herrick, 2000; Herrick, 2005), involving an early microvascular dysfunction. These observations raise the need for testing microvascular function in the finger pad in such patients. We and others have shown abnormal digital microvascular function in SSc patients, suggesting impaired endothelium-dependent vasodilation (Boignard et al., 2005; Murray et al., 2006) or abnormal neurovascular control (Roustit et al., 2008). However, few studies have focused on non endothelium-dependent function in SSc. To our knowledge, SNP iontophoresis has only been performed on the dorsal face of the finger of SSc patients (Anderson et al., 1999). However, clinically, SSc patients present digital ulcerations, which affect the finger pad in most cases (Guiducci et al., 2007). Thus, the lack of data about SNP iontophoresis on the finger pad is a limitation to the study of microvascular function in SSc patients.

In order to assess both endothelium and non endothelium-dependent vasodilation in a cohort of patients with SSc and correlate these to clinical outcomes, we first need to validate the SNP iontophoresis test on the finger pad compared to the forearm. This was the primary objective of this ancillary study. We thus performed SNP iontophoresis in SSc patients and in healthy subjects. The secondary objective of this study was to investigate whether the axon reflex is involved in the response, comparing the iontophoresis

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of SNP to that of sodium chloride, with and without lidocaine/prilocaine cream used as a pharmacological tool.

Patients and methods

Study population

This is an ancillary study of a larger cohort assessing microvascular function in SSc patients compared to healthy subjects. Six healthy controls were recruited through local newspaper advertisements. Inclusion criteria included age of 18 years or older, and no significant medical antecedents. Six patients with SSc were recruited from the Vascular Medicine Department. SSc was classified as limited (lSSc), limited cutaneous (lcSSc) or diffuse cutaneous SSc (dcSSc) using the criteria of LeRoy and Medsger (2001). Inclusion criteria included age of 18 years or older and the absence of any associated severe disease (diabetes mellitus, cancer, cardiac and/or pulmonary failure, pulmonary arterial hypertension, myocardial infarction, angina pectoris). If calcium-channel blockers were used to prevent Raynaud's phenomenon, they were stopped one week before enrolment. For all subjects, non-inclusion criteria included any allergies to local anesthetics 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 label pharmacology study. Upon arrival at the laboratory, subjects were placed in a temperature-controlled room (23 ± 1 °C). 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. For patients with SSc, fingers with active tip ulcerations were excluded. One hour before starting iontophoresis, 1 g of lidocaine/prilocaine cream (5 g tubes containing 125 mg lidocaine and 125 mg prilocaine) was applied to one skin site of the forearm and to one finger pad, as previously described (Cracowski et al., 2007; Roustit et al., 2008). The initial application of lidocaine/prilocaine cream covered 1 cm² of skin surface. An occlusive transparent dressing covering a larger skin area was placed over the cream to enhance cutaneous diffusion. The anesthetized area of skin was larger than the size of the local iontophoresis device. No cream was placed on the control sites. In order to avoid interference, the sites on the forearm were at least 3 cm apart.

One hour later, the lidocaine/prilocaine cream was removed with a cotton swab. After this acclimatization period, iontophoresis was performed, followed by local heating to reach maximal blood flow. The subjects were supine for the duration of the whole experiment, and blood pressure was monitored manually.

Iontophoresis protocol and laser Doppler measurements

Iontophoresis devices coupled to laser Doppler flowmetry probes with integrated local heaters (Perilont System, Perimed, Järfälla, Sweden) for the assessment of skin blood flow were fixed onto all the skin sites. Before beginning recording, the arm was immobilized with a vacuum cushion to ensure subject positioning (Fig. 1). In our practice, the use of this cushion decreases the artifacts associated with arm movement. A 5-minute baseline measurement was first recorded. All sites were then iontophoresed twice for 20 s at 200 μ A (with a 4-minute resting period between the two bursts) with 180 μ L of a 0.9% sodium chloride solution (NaCl). After return to baseline all sites were iontophoresed following the same protocol with a SNP solution (Nitriate[®], powder and solution for IV injection; 1 vial containing 50 mg of SNP diluted in 4 mL of water for injections). Cutaneous blood flux values were averaged over a 3-minute period at the iontophoresis plateau. When the plateau was not obvious, the flux was averaged

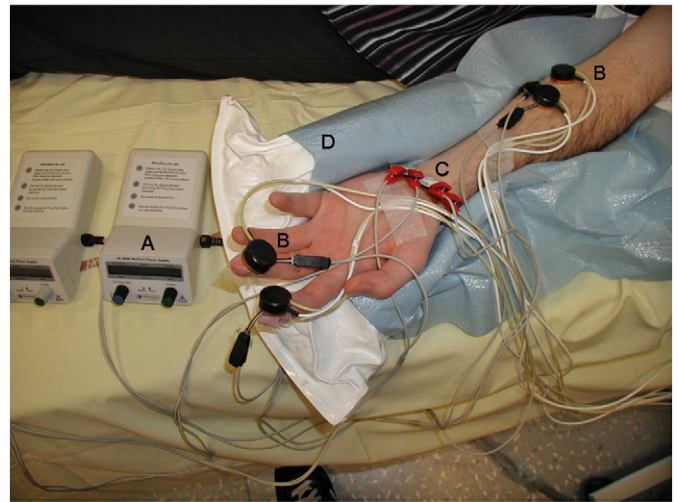


Fig. 1. Recording of digital cutaneous vascular conductance while performing sodium nitroprusside iontophoresis on the finger pad and the forearm. A: power supply; B: laser Doppler probes and drug delivery electrodes; C: dispersive electrodes; D: vacuum cushion.

between the 3rd and the 6th min after the second burst (which corresponds to the iontophoresis plateau on the forearm).

After return to baseline, thermal hyperemia was induced at all sites by local heating at 42 °C during 30 min followed by heating for 5 min at 44 °C to achieve maximal blood flow, as previously described (Cracowski et al., 2006; Salvat-Melis et al., 2006).

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 (Cracowski et al., 2006). Finally, CVC data were scaled to maximal vasodilatation (44 °C thermal plateau), as previously described (Roustit et al., 2008).

Statistical analysis

Quantitative data are expressed as the median and interquartile in parenthesis. Qualitative data are expressed as numerical values and percentage in parenthesis. Quantitative data were analyzed with the Wilcoxon test for paired analyses, with each subject serving as his/her own control. *p*-values less than 0.05 were considered statistically significant. The number of subjects was based on the assumption that more than 50% of the subjects would exhibit hyperemia following SNP iontophoresis on the finger pad. With 6 subjects by group, an exact binomial test with a nominal 0.05 one-sided significance level would have 89% power to detect the difference between the null hypothesis proportion (no subject with significant hyperemia) and the alternative proportion (50%) when the sample size is 6 (JD Elashoff; nQuery Advisor[®] for Windows v 6.01, Statistical Solutions, MA, USA).

Results

The demographic and clinical characteristics of the subjects included in this study are summarized in Table 1. Among patients, 2 were on calcium-channel blockers, 1 on angiotensin-converting enzyme inhibitors, 1 on angiotensin II receptor blocker, 1 on a beta-blocker and 1 on steroids.

In all healthy controls and patients with SSc, iontophoresis of SNP induced hyperemia in the forearm, (Table 2 and Fig. 2). The amplitude of this hyperemia was comparable between the two groups. The application of lidocaine/prilocaine cream did not affect the results of the iontophoresis of SNP in any subject. In contrast, no increase in CVC was observed in the finger pad in any healthy volunteer. SNP

iontophoresis induced clear-cut hyperemia in the finger pad in only one patient with SSc (ISSc, no sclerodactyly), without or with lidocaine/prilocaine cream pretreatment (Table 2 and Fig. 2). In the SSc group, 2 patients exhibited a weak tendency towards increased cutaneous conductance that was blunted by the lidocaine/prilocaine cream site (Fig. 2), suggesting non specific axon reflex hyperemia. Local heating induced hyperemia in all subjects at all sites. Median maximum conductances (interquartile) were not significantly different without and with lidocaine/prilocaine cream on the finger pad in healthy controls [49.6 (6.2) and 50 (34) mV/mm Hg, respectively] and in SSc patients [20.6 (24.7) and 28.6 (13) mV/mm Hg]. In contrast, lidocaine/prilocaine cream blunted the initial peak in healthy controls [43.2 (21.4) and 32 (33) mV/mm Hg; $p < 0.05$] but not in SSc patients [18.7 (23) and 21.8 (17) mV/mm Hg].

We also observed an increase of the CVC after the iontophoresis of NaCl on the forearm, approximately half the amplitude of that induced by SNP. Such vasodilation was blunted on the lidocaine/prilocaine cream site. However, NaCl-induced iontophoresis did not induce any vasodilation in the finger pad (Table 2).

In order to test whether the lack of a consistent effect of SNP iontophoresis on the finger pad was due to the iontophoresis protocol, we performed an additional experiment on four healthy volunteers with 200 μ A during 60 s, twice. We observed no increase in CVC in any of the 4 additional subjects.

We observed no side effects in any of the subjects enrolled, including no drop in humeral blood pressure.

Discussion

The iontophoresis of SNP leads to a consistent, non endothelium-dependent, increased CVC on the forearm, both in patients with SSc and healthy controls. This dilation is not due to an axon reflex, as the hyperemia was also observed on sites pretreated with lidocaine/prilocaine cream. However, on the finger pad, such dilation was observed only in one SSc patient and in no controls.

The iontophoresis technique is a method of non invasive transdermal drug delivery allowing the transfer of charged molecules using a low-intensity electric current. Combined with laser Doppler skin blood flow measurement, iontophoresis has a relatively low coefficient of variation (ranging from 12% to 40%) (Kalia et al., 2004) and is well tolerated. In animals, histopathic study of the skin after iontophoresis showed no micro-injury (Okuno et al., 2008). However, despite its widespread use, this technique may have technical and interpretative limitations. Indeed, the current applied to the skin may induce an axon reflex-dependent vasodilation in addition to the

Table 1
Demographic and clinical characteristics of healthy controls and patients with systemic sclerosis (SSc).

	Healthy controls (n = 6)	SSc (n = 6)
Age (years)	49.5 (5)	47.5 (16)
Female	4 (67)	4 (67)
Body mass index	24.4 (6.2)	24.8 (4.7)
Systolic/diastolic blood pressure	110.5 (10)/63.5 (13)	111.5 (30)/70.5 (21)
RP	0 (0)	6 (100)
RP: duration (years)	NA	17 (9)
RP: number of fingers involved	NA	10 (2)
RP: thumb involved	NA	5 (83)
RP: feet involved	NA	5 (83)
Disease duration (years)	NA	8.5 (9)
Digital pitting scars	NA	4 (67)
Sclerodactyly	NA	4 (67)
Rodnan-modified skin score	NA	5.5 (10)
ISSc/lcSSc/dcSSc	NA	2 (33)/2 (33)/2 (33)

Quantitative data were expressed as median (interquartile). Qualitative data were expressed as number (percentage). RP: Raynaud's phenomenon. NA: not applicable. ISSc: limited systemic sclerosis. lcSSc: limited cutaneous systemic sclerosis. dcSSc: diffuse cutaneous systemic sclerosis.

Table 2

Effects of the iontophoresis of sodium nitroprusside (SNP) and sodium chloride (NaCl) on the forearm and the finger pad of healthy controls and patients with systemic sclerosis (SSc), with or without lidocaine/prilocaine cream (l/p).

		Forearm		Finger pad		
		Baseline	Iontophoresis	Baseline	Iontophoresis	
HealthySNP controls (n = 6)	CVC	1 (0.7)	2.6 (0.9)*	10 (10.6)	7 (9.9)	
	%CVC _{max}	6 (3.4)	19.7 (38.9)*	20 (29)	14.2 (28.1)	
	SNP + l/p	CVC	1 (1.5)	3.8 (3.8)*	13.9 (10.5)	7.4 (10.5)
		%CVC _{max}	4.2 (3.2)	16.2 (69.3)*	21 (51.3)	11 (51.9)
	NaCl	CVC	0.7 (0.2)	1.5 (1.2)*	12.8 (15.7)	17.6 (11)
		%CVC _{max}	4.9 (7.5)	8.4 (8.8)*	22.4 (33.2)	38.8 (43)
NaCl + l/p	CVC	1.1 (0.3)	1.2 (1.1)	8.1 (12.7)	16.4 (7.9)	
	%CVC _{max}	6.1 (12.3)	6.3 (4.5)	12.2 (66.5)	33.5 (51.8)	
SSc patients (n = 6)	CVC	1 (0.9)	1.9 (1.8)*	3.5 (3.1)	5.3 (15.8)	
	%CVC _{max}	6.6 (6.4)	23 (30.3)*	11.4 (30)	21.9 (43.8)	
	SNP + l/p	CVC	1.3 (0.7)	2.9 (3.2)*	8.6 (6)	7.4 (6.8)
		%CVC _{max}	5.5 (9)	12.4 (22.9)*	34.7 (35.7)	45.4 (55.1)
	NaCl	CVC	0.7 (0.9)	1.3 (1.2)*	7.9 (6)	6.1 (8.7)
		%CVC _{max}	5.6 (7.5)	11.1 (6.3)*	25 (10.3)	30.1 (36.9)
NaCl + l/p	CVC	0.6 (0.3)	0.8 (0.5)	7.4 (11)	6.7 (8.9)	
	%CVC _{max}	2.8 (3.9)	4 (3.4)	41.8 (25.8)	44.4 (55.9)	

Data are expressed as median percentage (interquartile) of cutaneous vascular conductance (CVC, in mV/mm Hg) and percentage of the maximal vasodilation (% CVC_{max}).

* $p < 0.05$ versus baseline.

proper effect of the vasodilating substances (i.e. sodium nitroprusside) (Caselli et al., 2003; Droog et al., 2004). We actually observed an increase in CVC after iontophoresis of NaCl on the forearm. However, when pretreated with lidocaine/prilocaine, such vasodilation was not observed. This phenomenon is therefore attributable to a current-induced axon reflex vasodilation and is correlated to the intensity of the applied current (Tartas et al., 2004).

The absence of vasodilation after SNP iontophoresis on the finger pad is not due to technical problems related to the iontophoresis device, as we were able 1. to simultaneously induce consistent vasodilation in the forearm and 2. to subsequently evoke heating-induced vasodilation in the finger pad. We further tested whether a higher charge delivery iontophoresis protocol could increase CVC in the finger pad, with no positive result. Indeed, the second series of four healthy controls showed no effect of a three-fold increase in the iontophoresis time, suggesting that the doses (i.e. current intensity and iontophoresis length) initially chosen were not responsible for the absence of an effect. Although the only SNP-induced dilation occurred in a patient with no sclerodactyly, skin thickening on the digits of patients with SSc does not explain either the absence of such dilation in the finger pad. Indeed, similar results were observed in healthy controls and the two patients with no sclerodactyly. Moreover, two other patients presented skin thickening at the level of the forearm, and both were responders to the iontophoresis of SNP at this site. This further suggests that skin thickening is not a limitation to vasodilator therapeutic iontophoresis (Murray et al., 2008).

A potential explanation for the lack of hyperemia following digital SNP iontophoresis is an increased clearance of the drug. Indeed, capillary density in the finger pad is more than 100 times that of the forearm, and the exchange surface is even further increased by the numerous arteriovenous anastomoses. In addition, we observed in two SSc subjects a short non sustained hyperemia following SNP iontophoresis, differing from the sustained plateau observed in the forearm. Lastly, the only subject exhibiting a clear-cut dilation in the finger pad was a patient with SSc. In such a patient the lower capillary density may lead to decreased venous clearance, thus resulting in a higher interstitial SNP concentration. Therefore, one plausible explanation is that SNP is quickly cleared from the finger pad through

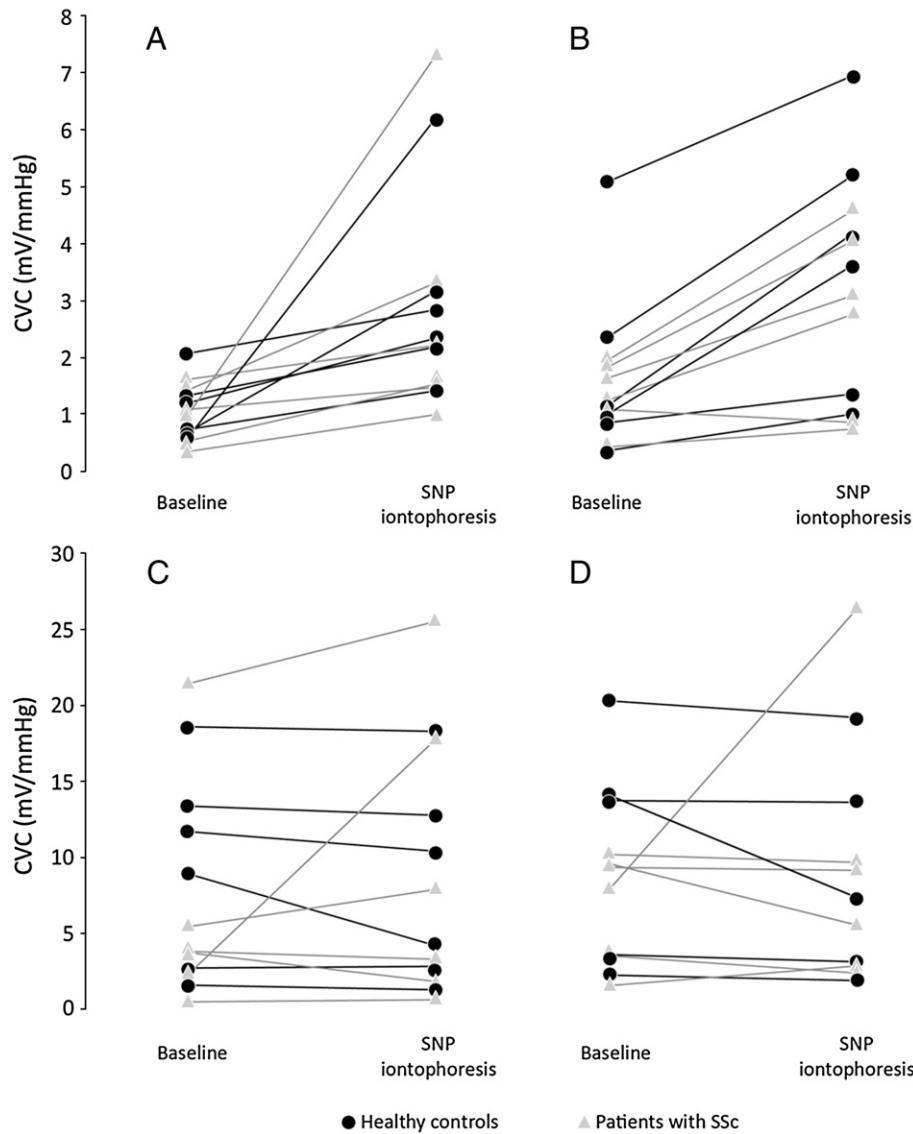


Fig. 2. Cutaneous vascular conductance (CVC) at baseline and after two bursts of SNP iontophoresis in healthy controls (black circles) and in SSc patients (grey triangles). Four locations were recorded: on the forearm without (A) and with (B) lidocaine/prilocaine pretreatment; on the finger pad without (C) and with (D) lidocaine/prilocaine pretreatment.

capillaries and subsequent venules. However, on the basis of the present study, we cannot conclude about this hypothesis.

In the present study, local thermal hyperemia was used as a reference for microvascular function assessment. It is of interest, that after lidocaine/prilocaine pretreatment we observed decreased amplitude of the peak hyperemia after local heating in healthy controls, in both the forearm and the finger. However, the anesthetic cream only affected the forearm CVC of patients with SSc. These data confirm previous work suggesting abnormal digital neurovascular control in such patients (Roustit et al., 2008).

In conclusion, the iontophoresis of SNP leads to a consistent, non axon reflex-dependent, increased cutaneous vascular conductance in the forearm, in both patients with SSc and healthy controls. In the finger pad however, such hyperemia was not consistent. As a consequence, we suggest that standard protocols used for SNP iontophoresis cannot be used on the finger pad as tools to assess non endothelium-dependent skin microvascular dilation. However, this does not rule out the potential interest of vasodilator iontophoresis as a treatment of digital ulcerations (Murray et al., 2008). Indeed, longer iontophoretic stimulation of the whole finger may be of clinical therapeutic interest.

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Correlation of Biomarkers of Endothelium Dysfunction and Matrix Remodeling in Patients with Systemic Sclerosis

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ABSTRACT. Objective. Systemic sclerosis (SSc) is a multisystem disease characterized by microvascular dysfunction and excessive fibrosis. However, the relationship between these 2 features remains unclear. Endothelial dysfunction can be assessed by quantifying plasma asymmetric dimethylarginine (ADMA), an endogenous inhibitor of endothelial nitric oxide synthase. Matrix remodeling can be assessed by quantifying serum tissue inhibitor of matrix metalloproteinases-1 (TIMP-1). Both biomarkers are elevated in patients with SSc. Our objective was to test whether plasma ADMA is correlated with serum TIMP-1.

Methods. We enrolled 91 subjects, 39 patients with SSc, 28 patients with primary Raynaud's phenomenon (RP), and 24 healthy volunteers. Plasma ADMA concentrations were measured by liquid chromatography-tandem mass spectrometry. Serum TIMP-1 concentrations were determined by ELISA.

Results. Mean ADMA concentrations were higher in patients with SSc ($0.68 \mu\text{M} \pm 0.12$) than in patients with primary RP or healthy volunteers (respectively, $0.56 \mu\text{M} \pm 0.14$ and $0.62 \mu\text{M} \pm 0.12$; $p = 0.002$). Median serum TIMP-1 concentrations were increased in patients with SSc compared to primary RP and healthy volunteers [12 (9–15), 11 (8–13), and 10 (7–13) nM, respectively; $p = 0.05$]. In the SSc group, we observed a statistically significant correlation between plasma ADMA and serum TIMP-1 ($r = 0.34$, $p = 0.035$).

Conclusion. These data are consistent with our hypothesis of an association of endothelial dysfunction and matrix remodeling in scleroderma spectrum disorders. (First Release April 1 2009; *J Rheumatol* 2009;36:984–8; doi:10.3899/jrheum.080924)

Key Indexing Terms:

SYSTEMIC SCLEROSIS

MATRIX METALLOPROTEINASES

ASYMMETRIC DIMETHYLARGININE

MATRIX REMODELING

ENDOTHELIUM

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Microvascular dysfunction is an early event in the pathogenesis of systemic sclerosis (SSc). It is characterized by an increased lymphocyte binding as well as a decreased endothelium-dependent flow-mediated relaxation¹. Endothelial dysfunction can be investigated by functional testing² or by measurement of biomarkers. Recently, asymmetric dimethylarginine (ADMA), an endogenous inhibitor of endothelial nitric oxide (NO) synthase, has emerged as a promising biomarker of endothelial dysfunction in cardiovascular diseases³⁻⁵. Further, plasma ADMA is increased in patients with SSc when compared to patients with primary Raynaud's phenomenon (RP)^{6,7}.

SSc is characterized by an increased collagen deposition. The net accumulation of extracellular matrix is the result of the balance between synthesis and degradation of extracellular components, partly regulated by enzymes called matrix metalloproteinases (MMP). The tissue fibrosis observed in SSc may be due to an inhibition of MMP activity by MMP-specific inhibitors called tissue inhibitors of metalloproteinases (TIMP). Serum concentration of MMP-1 is increased in patients with SSc^{8,9}. Both MMP-1 and MMP-9

are inhibited by TIMP-1, and serum concentrations of TIMP-1 are consistently high in SSc⁸⁻¹¹.

While microvascular dysfunction and matrix remodeling have been widely assessed in SSc, there is a lack of data about the pathophysiological link between the 2 processes. Our objective was to test whether ADMA, a biomarker of endothelial dysfunction, is correlated to TIMP-1, a biomarker of matrix remodeling.

MATERIALS AND METHODS

Study population. This was a descriptive single-center case-control study performed as described¹². All subjects gave written informed consent, and the study was approved by the institutional review board of Grenoble University Hospital, France, in January 2004. This study is part of a larger cohort study about the vascular phenotype enrolling patients with SSc, patients with primary RP, and healthy volunteer controls. Inclusion and exclusion criteria are described elsewhere¹².

Laboratory evaluations. Determination of ADMA and symmetric dimethylarginine (SDMA): prechilled EDTA vacutainers were used to collect blood samples and immediately placed on ice. They were centrifuged at 4°C within a few minutes and plasma samples were stored at -80°C until analysis. Then, all samples were sent in dry ice by an express carrier to the Institute of Experimental and

Clinical Pharmacology and Toxicology (Hamburg, Germany). ADMA concentrations were measured by liquid chromatography-tandem mass spectrometry, as described and validated¹³.

ELISA for serum MMP-1, MMP-9, and TIMP-1: samples were centrifuged at 1800 g for 10 min at 15°C, serum was stabilized with a protease inhibitor cocktail without EDTA (Roche Diagnostic, Meylan, France), and aliquots were stored at -80°C. Serum TIMP-1 concentrations were determined by a commercial ELISA assay (Amersham Pharmacia Biotech, Buckinghamshire, UK). This assay quantifies both free and bound TIMP-1 with a detection limit of 1.25 ng/ml. Serum MMP-1 and MMP-9 concentrations were also evaluated by commercial ELISA (R&D Systems, Abingdon, UK). The minimum detectable levels were 0.021 ng/ml for proMMP-1 and 0.516 ng/ml for total MMP-9.

Laser Doppler measurements. Cutaneous blood flow was assessed using laser Doppler flowmetry (Periflux System 5000; Perimed, Järfälla, Sweden) as described¹³. The amplitude of the post-occlusive reactive hyperemia response was expressed as peak cutaneous vascular conductance raw value in mV/mm Hg.

Data analysis. The number of subjects was calculated on the hypothesis that we would detect a correlation coefficient of 0.4 between ADMA and TIMP-1, with a power of 80%, and an alpha risk of 0.05 for a 1-tailed analysis.

Data distribution was analyzed prior to quantitative data analysis. When data did not follow a normal distribution, nonparametric

Table 1. Demographic, clinical, and biological characteristics of patients with cutaneous systemic sclerosis (SSc), primary Raynaud's phenomenon (RP), and healthy controls (HC).

	Controls, n = 24	RP, n = 28	SSc, n = 39
Mean age, yrs (SD)	52 (10)	49 (9)	52 (11)
Female, n (%)	21 (87)	25 (89)	35 (90)
Raynaud's phenomenon, n (%)	0 (0)	28 (100)	39 (100)
Median Raynaud's disease duration, yrs, (10th-90th percentiles)	0 (0)	14 (5-42)	10 (3-27)
RP, median no. of fingers involved (10th-90th percentiles)	0 (0)	8 (6-10)	10 (8-10)
RP, thumbs involved, n (%)	0 (0)	14 (50)	31 (80)
RP, feet involved, n (%)	0 (0)	13 (46)	30 (77)
Median SSc disease duration, yrs (10th-90th)	NA	NA	5 (1-16)
Digital pitting scars (%)	0 (0)	0 (0)	22 (56)
Sclerodactyly, n (%)	0 (0)	0 (0)	29 (74)
Median Rodnan modified skin score (10th-90th)	0 (0)	0 (0)	6 (0-24)
ISSc/lcSSc/dSSc, n	0/0/0	0/0/0	8/22/9
Pulmonary fibrosis, n (%)	0 (0)	0 (0)	11 (28)
Pulmonary arterial hypertension, n (%)	0 (0)	0 (0)	1 (2)
Esophageal dysmotility, n (%)	0 (0)	0 (0)	21 (54)
Mean creatinine clearance, ml/min (SD)	86 (22)	79 (12)	91 (25)
Mean microalbuminuria, mg/l (SD)	15 (11)	17 (19)	16 (17)
Mean cardiac rate, beat/min (SD)	62 (10)	67 (13)	71 (13)
Mean systolic/diastolic blood pressure, mm Hg (SD)	113 (13)/66 (9)	110 (14)/66 (9)	119 (20)/69 (11)
Mean body mass index, kg/cm ² (SD)	24 (4)	20 (2)	23 (3)
Autoantibodies			
Anticentromere, n (%)	0 (0)	0 (0)	16 (41)
Antitopoisomere I, n (%)	0 (0)	0 (0)	11 (28)
Mean plasma LDL cholesterol, g × l ⁻¹ (SD)	1.11 (0.3)	1.03 (0.3)	1.11 (0.3)
Mean plasma glycemia mmol × l ⁻¹ (SD)	4.6 (0.6)	4.6 (0.9)	4.6 (0.5)

NA: not applicable; ISSc/lcSSc/dSSc: limited/limited cutaneous/diffuse systemic sclerosis; LDL: low density lipoprotein.

statistical methods were performed. *p* values less than 0.05, corrected for multiple comparisons, were considered significant. Quantitative data were expressed as the mean \pm standard deviation or median and 10th and 90th percentiles.

RESULTS

The characteristics of the 91 patients enrolled in our study are listed in Table 1.

Mean ADMA concentrations were significantly higher in patients with SSc than in patients with primary RP or controls (0.68 ± 0.12 , 0.56 ± 0.14 , and 0.62 ± 0.12 μM , respectively; $p = 0.002$; Figure 1). Mean SDMA concentration was also significantly higher in patients with SSc than in patients with primary RP or controls (0.59 ± 0.12 , 0.54 ± 0.10 , and 0.53 ± 0.11 μM ; $p = 0.038$; Figure 1). No difference was shown in ADMA and SDMA concentrations between the different SSc subsets. No significant difference was observed between controls and patients with primary RP.

Median TIMP-1 concentration was higher in patients with SSc [12 nM (range 9–15)] than in patients with primary RP [11.7 nM (8–13)] and controls [10.1 nM (7–13)] ($p = 0.05$; Figure 2). Similarly, median MMP-1 levels were higher in patients with SSc [0.09 nM (0.04–0.20)] than in controls [0.05 nM (0.03–0.11)] ($p = 0.0263$; Figure 2). There was no difference in MMP-9 level between patients with SSc, patients with primary RP, and controls [4.61 nM

(2.79–9.31), 4.16 nM (2.56–7.48), and 4.54 nM (3.15–6.19)].

In the SSc group, we observed a statistically significant correlation ($r = 0.34$; $p = 0.034$) between ADMA, a biomarker of endothelial dysfunction, and TIMP-1, a biomarker of matrix remodeling (Figure 3). No correlation between ADMA or TIMP-1 and Rodnan skin score, scleroderma subset, pulmonary status, or autoantibody status was found.

Post-occlusive reactive hyperemia peak CVC was inversely correlated with serum TIMP-1 concentration ($r = -0.36$; $p = 0.004$) but not with ADMA. Moreover, TIMP-1 concentration was higher in patients with [12.1 nM (9.5–15.2)] versus those without [10.4 nM (7.7–13.7)] digital pitting scars ($p = 0.026$). We also observed a nonsignificant trend towards higher ADMA concentration in patients with digital pitting scars.

DISCUSSION

Rajagopalan, *et al* showed that ADMA was increased in patients with secondary RP compared to primary RP⁶. Similar results were obtained by Dooley, *et al*, who also demonstrated that ADMA concentrations in patients with primary RP were not different from those of healthy controls⁷. Further, Dooley, *et al* showed that patients with diffuse cutaneous SSc exhibited increased ADMA concentration, whereas it was similar to controls in patients with

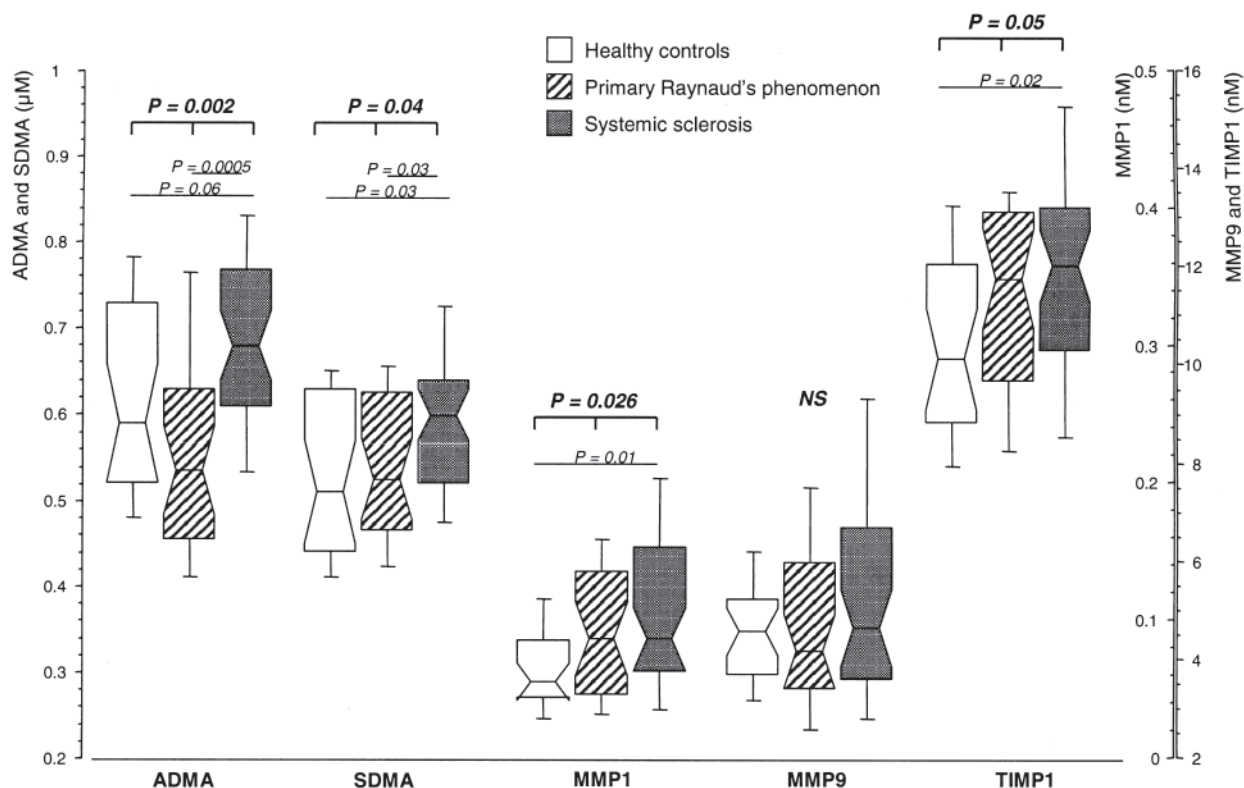


Figure 1. Plasma ADMA, plasma SDMA, serum MMP-1, serum MMP-9, and serum TIMP-1 concentrations in patients with SSc, primary Raynaud's phenomenon, and controls.

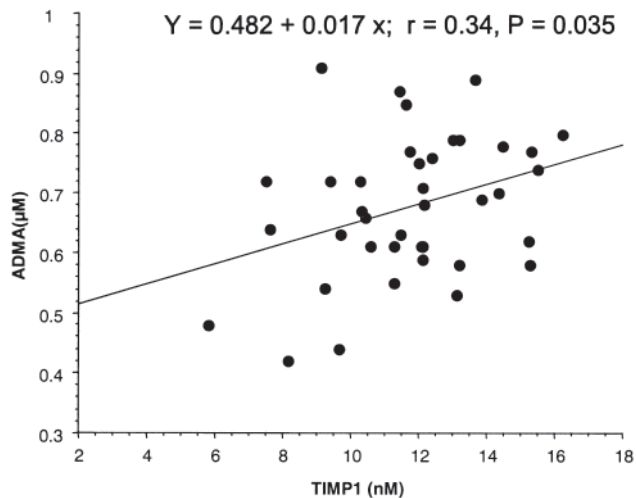


Figure 2. Correlation between plasma ADMA and serum TIMP-1 concentrations in 39 patients with SSc.

limited SSc⁷. In contrast, we observed no effect of disease stage on ADMA concentration in our study. Taken together with the observation that creatinine clearance was similar in controls and in patients with SSc, this suggests that the observed increase in ADMA concentrations is not due to an abnormal renal function.

Several studies showed that TIMP-1 levels are consistently high in patients with SSc, and may be associated with disease activity⁸⁻¹¹. Thus, our finding of increased TIMP-1 serum concentration in the SSc group agrees with previous results.

While serum MMP-1 concentration was low in healthy controls, higher values were observed in the SSc group, while no significant difference was observed between SSc and primary RP patients. Studies of lung fibrosis support the notion that MMP-1 is upregulated in fibrotic diseases¹⁴. Thus, our study provides further evidence of a paradox whereby excessive accumulation of collagen in fibrotic disease is associated with an overexpression of the cleaving enzyme, and not absence of it, as might have been expected.

Divergent results have been observed concerning serum MMP-9 concentrations^{10,15}, suggesting that quantification of MMP-9 is not a useful biomarker in SSc.

From a pathogenic point of view, SSc is characterized by an early endothelial-dependent vasodilatation dysfunction, and an increased lymphocyte transmigration, leading to increased production of cytokines¹. The latter modulates MMP and TIMP expressions. We characterized a correlation between ADMA, a biomarker of endothelial dysfunction, and TIMP-1, a biomarker of matrix remodeling, in SSc. In addition, serum TIMP-1 concentration was inversely correlated to post-occlusive reactive hyperemia, and was increased in patients with digital pitting scars. The link between NO and MMP in SSc remains unclear. NO inhibits collagen secretion in normal fibroblasts, but such regulation

is lost in SSc fibroblasts. It has been suggested that this phenomenon may be due to the upregulation of MMP-1 and/or inhibition of prolyl hydroxylase¹⁶. Another explanation implicated anti-fibroblast antibodies in SSc that may increase collagenolytic activity and MMP-1 production in dermal fibroblasts¹⁷. More functional experiments are required to provide a definitive conclusion, especially in the different subgroups of SSc.

We showed for the first time that in patients with SSc, ADMA, a biomarker of endothelial dysfunction, was increased and was positively correlated with TIMP-1, a biomarker of matrix remodeling. Further, we observed a correlation between TIMP-1 concentration and a global test of microvascular dysfunction. These data suggest an association between endothelial dysfunction and matrix remodeling in scleroderma spectrum disorders. However, further studies are required to test whether there is a direct pathophysiological link.

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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.

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

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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 enrollment 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 ($24 \pm 1^\circ\text{C}$). 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.

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 $\% \text{BL} \cdot \text{s}$) 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

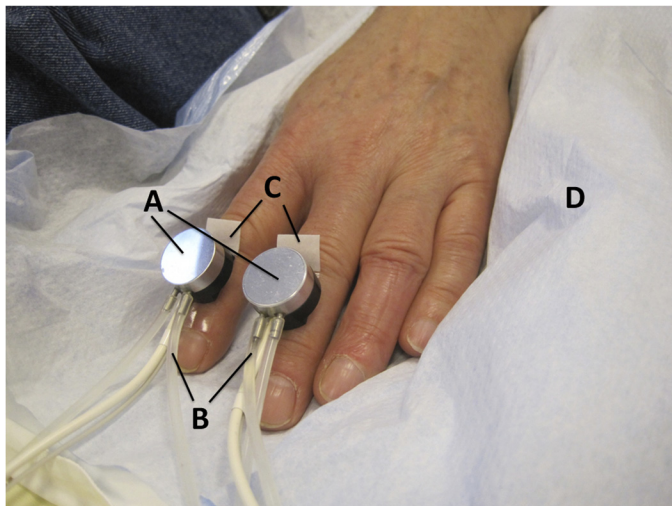


Fig. 1. Single-point laser Doppler probes (A) with a liquid cooling system made of silicon tubes (B). C: probes were fixed with double-sided tape. D: a vacuum cushion was used to decrease artifacts associated with arm movements.

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) $\mu\text{mol/l}$, 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).

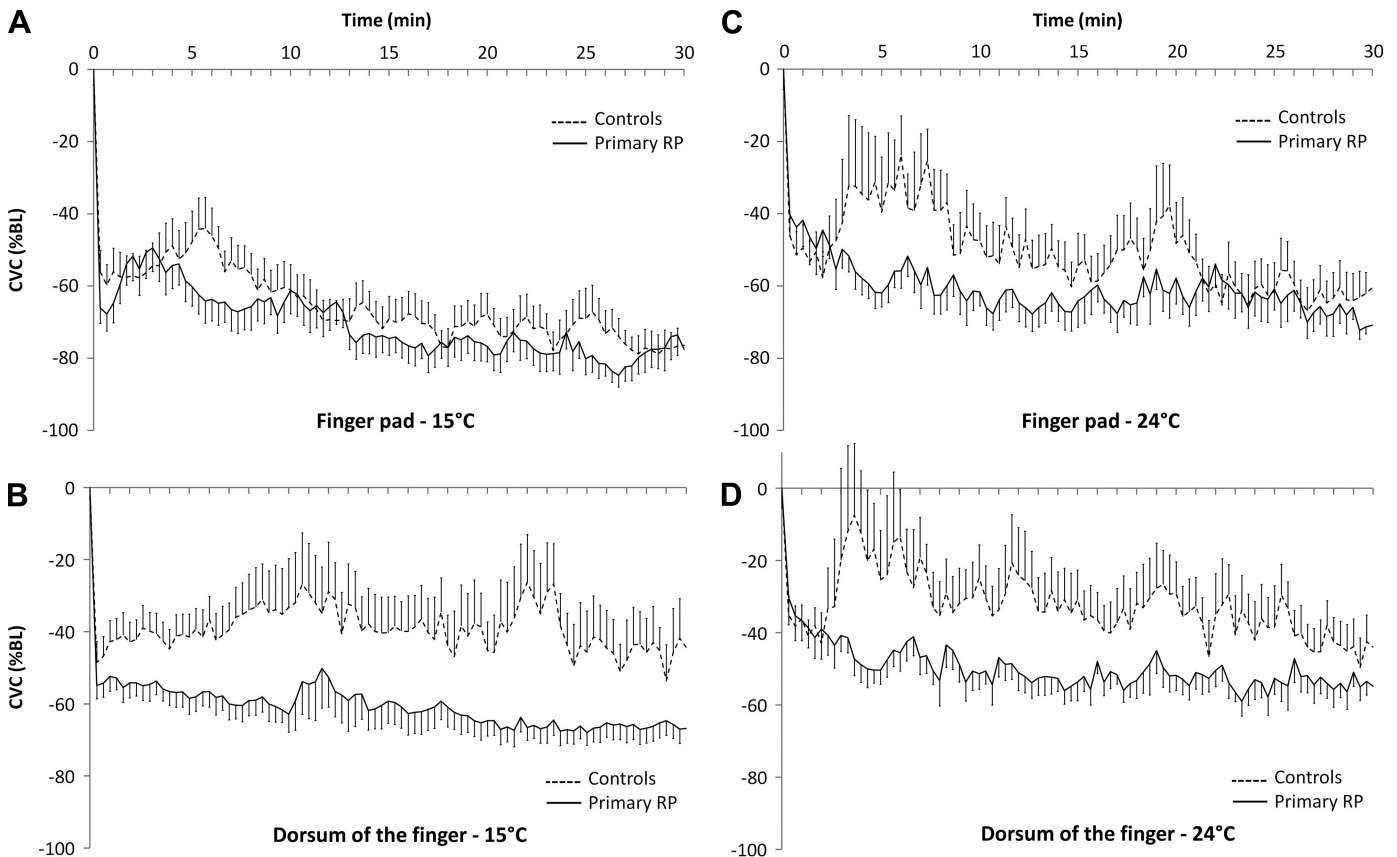


Fig. 2. Mean (SE) cutaneous vascular conductance (CVC; expressed as a percentage decrease from baseline CVC) in participants with primary Raynaud's phenomenon (RP; plain lines) and controls (dash lines). Local cooling to 15°C (A and B) and 24°C (C and D) was performed on the finger pad (A and C) and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

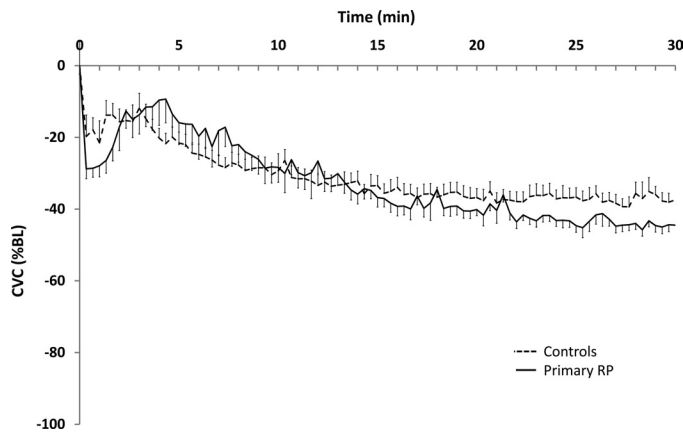


Fig. 3. Mean (SE) CVC (expressed as a percentage decrease from baseline CVC) in participants with primary RP (plain line) and controls (dash line) while cooling to 15°C on the forearm.

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 (AA_{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 (AA_{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 (AA_{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 (AA_{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 (AA_{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 (AA_{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 (AAC₀₋₃₀). 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 CO₂ 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 (AAC₀₋₃₀). 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.

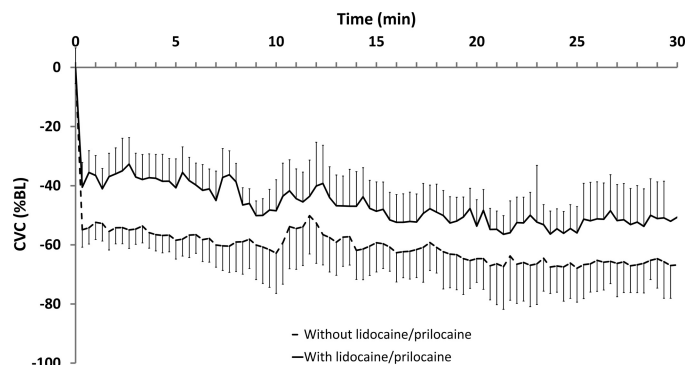


Fig. 4. Mean (SE) CVC (expressed as a percentage decrease from baseline CVC) in participants with primary RP while cooling to 15°C on the dorsum of the finger with (plain line) and without (dash line) lidocaine/prilocaine pretreatment.

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 paroxysmic 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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DISCLOSURES

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

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DISCUSSION

Ce travail a consisté à mettre en place différents protocoles au stade animal et volontaires sains pour tester l'efficacité de la iontophorèse thérapeutique avec des molécules ayant des propriétés vasodilatatrices. L'objectif à terme est de proposer ce traitement chez des patients ayant une atteinte microvasculaire cutanée, la ScS étant potentiellement le modèle applicatif pathologique, la iontophorèse chez les patients ScS ayant déjà été réalisée à des fins physiologiques et non thérapeutiques.

La iontophorèse de SNP a été réalisée chez des patients ScS (comparés à des volontaires sains) comme technique pour évaluer la fonction microvasculaire cutanée non endothélium dépendante (Roustit *et al.*, 2009). Elle induit une hyperhémie au niveau de l'avant-bras qui n'est pas affectée par le pré traitement par la lidocaïne /prilocaïne (permettant d'exclure le biais d'une réponse axonale réflexe), par contre, il n'a pas été observée d'augmentation de la conductance cutanée vasculaire au niveau de la pulpe du doigt. Cette méthode n'est donc pas applicable pour l'exploration de la pulpe du doigt.

Les rares essais de iontophorèse thérapeutique dans la sclérodémie n'ont pas montré d'efficacité. Shetty a publié une étude pilote chez trois patients sclérodermiques traités avec une iontophorèse d'acide acétique de 2 à 5 % à 100 μ A pendant soit 8 minutes soit 20 minutes suivie d'un traitement par ultra sons de 1.5 W/cm², cette combinaison de traitement ayant été testée dans des tendinites calcifiantes (Shetty *et al.*, 2005). Le critère de l'étude était la réduction des calcifications au contrôle radiologique et l'étude concluait à l'absence de bénéfice et l'absence d'effet secondaire, néanmoins, la piste de la iontophorèse thérapeutique devait être poursuivie avec d'autres molécules plus pertinentes et/ou des objectifs clairs (amélioration de la microcirculation, amélioration des UD...)

Trois voies pharmacologiques ont été étudiées dans notre travail: celle du NO, celle des anti-endothélines et celle des prostacyclines. L'association d'une iontophorèse d'une molécule avec un vasodilatateur per os pourrait être une piste intéressante, elle permet surtout de pouvoir proposer à termes des futurs protocoles de recherche clinique testant des iontophorèses cutanées avec des molécules vasodilatatrices chez des patients avec déjà un traitement per os dans le cadre de leur pathologie. La bonne tolérance constatée dans le cadre de l'étude INFLUX-VS permet de ne pas contre-indiquer l'association d'une iontophorèse avec le SNP avec le sildénafil per os, bien qu'une surveillance, notamment systémique tensionnelle, soit à prévoir. Le SNP n'est néanmoins pas la meilleure molécule candidate du fait de sa demi-vie très courte ne permettant pas d'envisager une efficacité suffisante tant sur la dilatation microvasculaire attendue (effet trop bref), que sur l'aspect pragmatique de la iontophorèse thérapeutique (les séances de traitement pour le patient seraient trop longues ou trop fréquentes). L'effet vasodilatateur au niveau de la microcirculation du sildénafil per os existe mais n'est pas significatif cliniquement sur le flux basal (Blaise *et al.*). Cependant, ce traitement est actuellement en évaluation pour le phénomène de Raynaud et la prise en charge des ulcérations digitales.

En ce qui concerne l'efficacité recherchée en termes d'action vasodilatatrice locale, les conclusions des différents protocoles orientent vers la recherche d'une molécule candidate optimisée en iontophorèse cutanée plus qu'à l'association d'une iontophorèse cutanée avec un traitement systémique. Les facteurs influençant l'efficacité d'une iontophorèse sont nombreux et ont été préalablement exposés (pH, pKa etc...). La stratégie a consisté à sélectionner les molécules vasodilatatrices étant déjà utilisées dans des atteintes vasculaires de la ScS, notamment l'HTAP : les anti-endothélines, les PDE 5 et la prostacycline et analogues de prostacyclines. Après l'étude du screening de la plupart de ces molécules en iontophorèse chez

le rat (INFLUX-RAT), les deux meilleures molécules candidates retenues en termes d'efficacité étaient le tréprostinil et l'iloprost (Blaise *et al.*). Ce résultat est cohérent puisque ces deux molécules sont des acides faibles ionisées à pH neutre (pKa inférieur au pH). L'ensemble des molécules testées en iontophorèse était bien tolérée d'un point de vue systémique et local chez le rat.

Les résultats encourageant d'INFLUX-RAT ont permis la mise en place et la réalisation de l'étude avec ces deux molécules chez les volontaires sains (Etude INFLUX-IT-VS). L'iloprost a été rapidement abandonné du fait d'une toxicité locale (brûlure de grade 1), l'hypothèse immunoallergique étant peu probable après enquête et avis allergologique. La molécule restant était le tréprostinil qui a montré une vasodilatation significative et soutenue, permettant de déterminer clairement la concentration nécessaire dans la solution pour obtenir un effet vasomoteur significatif. Cette concentration de 250 µM correspond à 1/10ème de la concentration la plus faible (1mg/mL) utilisée par voie sous cutanée dans l'HTAP. Une deuxième partie du protocole était prévue dès le départ pour affiner la pharmacodynamie en cas d'efficacité de la iontophorèse de tréprostinil. Une surveillance jusqu'à 24 heures a donc été réalisée après les iontophorèses de tréprostinil permettant de voir un effet vasodilatateur soutenu de la iontophorèse du tréprostinil jusqu'à 8 à 9 heures après la iontophorèse avec une bonne tolérance cutanée locale ainsi qu'une bonne tolérance systémique.

Il reste néanmoins de nombreuses questions en suspens avant d'envisager ce traitement à des patients malades, notamment des patients ScS:

1/ L'action de la iontophorèse cutanée avec un principe actif induit-elle bien une action principale loco-régionale au niveau dermique notamment et non principalement systémique ?

L'absence d'effet secondaire systémique et la dilatation locale en LDI orientent vers une action principalement locale, mais les études jusqu'à présent réalisées sont restées non invasives et sans explorations notamment dermiques. Le protocole TIPPS (Treprostinil Iontophoresis: a Pharmacokinetic and Pharmacodynamic Study) a été rédigé et devrait être débuté dernier trimestre 2011 pour répondre à ces questions. TIPPS correspond en la réalisation de iontophorèses cutanées de tréprostinil selon les mêmes conditions qu'INFLUX IT VS (même intensité et durée de iontophorèse et même concentration de tréprostinil), mais avec une surface plus importante (7,2 cm²) cohérente avec la cible thérapeutique (ulcérations), au niveau de l'avant-bras, de la pulpe et de la face dorsale des doigts chez le volontaire sain mais avec des compléments d'analyses pharmacocinétique et pharmacodynamique. L'étude prévoit ainsi de quantifier le passage systémique avec des dosages sanguins de tréprostinil ainsi que de quantifier le passage en dermique avec des dosages de tréprostinil après insertion de fibres de microdialyse cutanées (pour les iontophorèses au niveau de l'avant-bras). L'effet global bénéfice/risque devra être étudié par rapport aux effets systémiques versus l'effet local.

2/ La iontophorèse est-elle reproductible ?

Pour répondre à cette question, au moins quatre points sont à discuter : la reproductibilité des techniques d'acquisition du signal, la reproductibilité intra et inter individuelle, la reproductibilité spatiale (essentiellement avant-bras, pulpe ou dos du doigt), la reproductibilité spécifique de la iontophorèse au tréprostinil.

- L'équipe travaille déjà actuellement sur les questions de **reproductibilité des techniques d'acquisition des signaux en microcirculation** (cf Chapitre 5.1). L'ensemble des protocoles actuellement en cours ou futur seront réalisés avec le LDI et/ou le LSCI. Les limites techniques rencontrées notamment pour réaliser le test d'hyperhémie post chauffage avec des

sondes de LDI a imposé dans certains protocoles l'utilisation en parallèle du LDF. L'état des connaissances actuelles ayant déjà mis en évidence, l'efficacité de la iontophorèse de tréprostinil avec les tests de références, le LDF n'aura plus besoin d'être utilisé sur cette thématique précise. L'équipe a notamment démontré que dans la plupart des conditions d'études, une mauvaise résolution spatiale du LDF était rédhibitoire pour une utilisation clinique en dehors de certains sites comme la pulpe digitale plus richement perfusés (Roustit *et al.*, 2010a; Roustit *et al.*, 2010b).

Le LDI quand à lui ne permet pas de combiner une bonne résolution spatiale et temporelle. L'outil n'est de toutes les manières pas conçu dans ce but. La reproductibilité du LSCI est nettement meilleure que celle obtenue en LDF (*figure 24*).

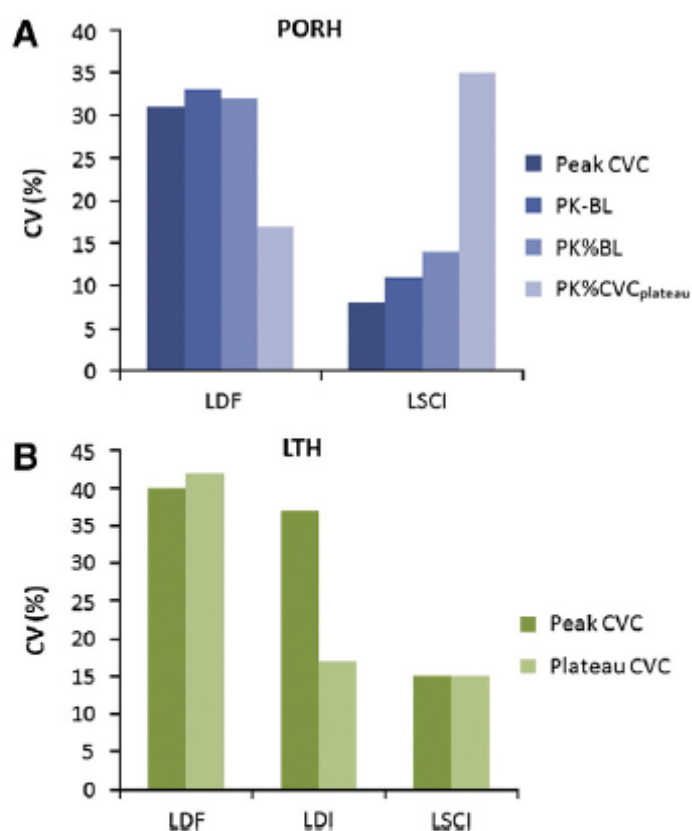


Figure 24 :
Exemple de coefficient de variation de la reproductibilité intra sujets en LDF, LDI et LSCI pour la PORH et la LTH. (Roustit *et al.*, 2010b)

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.

Compte tenu d'une mise sur le marché assez récente du LSCI, une seule étude a pour l'instant été réalisée dans le domaine de la sclérodémie. Andrea Murray a utilisé une version initiale du LSCI de la firme Moor comparée à la videocapillaroscopie sur 3 patients et trouve des résultats décevants pour l'exploration du lit capillaire unguéal (Murray *et al.*, 2009). Cependant, après l'avoir testé, nous avons à la même époque renoncé à travailler avec cet outil inadapté à la mesure de larges champs à hautes vitesses. Nous avons récemment montré que la reproductibilité de cet outil pour évaluer des tests dynamiques tels que l'hyperhémie

postocclusive est excellente (Roustit *et al.*, 2010b). De plus, l'équipe du Pr Pierre Abraham vient de montrer que le LSCI permet d'obtenir une quantification de l'hyperhémie postocclusive avec des coefficients de variation excellents à condition de tirer avantage de l'acquisition bidimensionnelle de l'outil, c'ad, en prenant des zones d'intérêt de plus de 10 mm² (Rousseau *et al.*, 2011a; Rousseau *et al.*, 2011b), ce qui est systématiquement notre cas. Depuis, nous avons également testé et évalué la reproductibilité du LSCI développé par la firme Perimed et validé la linéarité de son signal (Millet *et al.*). Enfin, l'équipe du Pr Abraham vient de montrer que le LSCI peut être utilisé sans altération des résultats dans un intervalle de 10 à 30 cm de la surface étudiée (Mahe *et al.*). Le LSCI permettant une acquisition en large champ avec une résolution temporelle très fine paraît extrêmement prometteur pour l'étude de la microcirculation cutanée. Cet outil est donc particulièrement adapté à l'étude très fine de variations de flux cutané très rapide et devrait rapidement remplacer la fluxmétrie laser Doppler.

Une autre discussion qui pourrait être abordée est le **mode d'expression des données**. Elle intervient dans la reproductibilité et les valeurs doivent être exprimées dans les unités les plus reproductibles que ce soit avec le LDF, le LDI ou le LSCI selon les topographies explorées (*figure 24*).

Dans les études, les résultats peuvent être exprimés en unités de perfusion (unité arbitraire) ou mieux en conductance vasculaire cutanée (CVC), qui prend en compte les variations de pression artérielle (O'Leary, 1991). Les résultats peuvent également être exprimés en CVC % BL qui tient compte également de l'état vasculaire initial. L'analyse des résultats est faite avec le logiciel PimSoft après sélection et détermination de la taille de la région d'intérêt (ROI). L'amplitude de la réponse à l'iontophorèse est également évaluée en aire sous la courbe (AUC) pour les études de pharmacodynamie.

- En ce qui concerne les reproductibilités intra et inter individuelles, la littérature rapporte une bonne reproductibilité de la iontophorèse d'acétyl choline et de SNP avec le LDI (Jadhav *et al.*, 2007). Le mode d'expression des data est néanmoins toujours important à préciser puisque dans cette étude, la perfusion est corrigée par une intégrale de la résistance cutanée. Dans notre étude INFLUX-VS, la reproductibilité intra individuelle à 7 jours du pic de SNP avec le LDI était de 22 % de coefficient de variation avec un coefficient de corrélation intra classe de 0.72 (Blaise *et al.*). Les études de reproductibilité avec le LDF avec la iontophorèse d'acétyl choline ne sont pas bonnes (de 25% à 35% selon l'expression des data) (Agarwal *et al.*). La reproductibilité de la iontophorèse de SNP avec le LDF est encore moins bonne. Une étude non publiée de l'équipe chez 14 sujets retrouve des CV allant de 69 à 160 % sur le dos du doigt et de 63 % à 95 % sur l'avant-bras selon le mode d'expression des data.

Ces résultats renforcent donc l'intérêt de l'utilisation du LDI par rapport au LDF.

- La iontophorèse a-t-elle **une bonne reproductible spatiale** (essentiellement sur l'avant-bras, pulpe ou dos du doigt)? On sait les réponses variables et le problème de la reproductibilité de la iontophorèse entre l'avant-bras, le dos du doigt et la pulpe du doigt? (Roustit *et al.*, 2009; Roustit *et al.*) Il peut être discuté une variation de l'effet de la iontophorèse selon la topographie cutanée, probablement du fait de la variation du lit vasculaire en fonction des différents territoires étudiés. Certains résultats de mauvaises reproductibilités intra ou inter individuelles pourraient être expliqués par cette mauvaise reproductibilité spatiale (référence à l'étude non publiée de l'équipe chez 14 sujets retrouvant des CV de la iontophorèse de SNP avec le LDF allant de 69 à 160 % sur le dos du doigt et de 63 % à 95 % sur l'avant-bras selon le mode d'expression des data.)

L'objectif du projet étant de développer un outil thérapeutique chez le patient ScS, la question qui nous intéresse porte donc surtout sur l'effet thérapeutique au niveau des doigts, atteinte privilégiée de ces patients. Une partie de la réponse sera sans doute élucidée lors de la phase secondaire du protocole TIPPS qui prévoit l'inclusion de patients sclérodermiques où l'on pourra décrire les réponses des iontophorèses au niveau digital avec atteinte microvasculaire.

- En ce qui concerne la **reproductibilité intra et inter individuelle de la iontophorèse digitale de tréprostinil**, un protocole spécifique d'étude des coefficients de variabilité avec la iontophorèse de tréprostinil n'a pas été mis en place. On sait que les résultats de reproductibilité de iontophorèse sont variables selon la molécule utilisée. Les résultats cohérents entre la première et la deuxième partie d'INFLUX-IT-VS vont dans le sens d'une bonne reproductibilité même si une conclusion ne peut être portée. Le protocole TIPPS étant construit avec les mêmes modalités de iontophorèse de tréprostinil (durée, concentration..), il pourra également répondre à cette question. La lourdeur des protocoles nous incite à attendre l'analyse intermédiaire de TIPPS pour connaître la topographie la plus satisfaisante en termes de résultat. L'idée est de ne pas tester la reproductibilité sur l'ensemble des sites, notamment si la iontophorèse de tréprostinil au niveau de la pulpe n'entraîne pas de vasodilatation significative chez le volontaire sain.

3/ L'action vasodilatatrice chez le volontaire sain sera-t-elle constatée chez les patients malades ?

De nombreux biais peuvent intervenir. La réponse ne pourra bien entendu être fournie que si la première partie de TIPPS chez le volontaire sain permet d'autoriser la deuxième, à savoir la iontophorèse au chez le patient ScS sans UD. Après une analyse chez 10 volontaires sains et en cas d'analyse intermédiaire favorable, une deuxième partie est en effet d'ores et déjà prévue dans l'étude TIPPS avec l'analyse de l'effet pharmacodynamique de la pulpe ou de la face dorsale des doigts chez des patients sclérodermiques.

Les résultats favorables d'INFLUX IT-VS permettent d'espérer une réponse positive chez les patients ScS, mais la réponse à la iontophorèse peut être influencée par les modifications physiopathologiques de la maladie.

La iontophorèse pourrait être influencée par les conditions physiques locales notamment vasculaires et fibrosantes. L'atteinte fonctionnelle vasculaire ou la raréfaction vasculaire chez le patient ScS pourraient induire une vasodilatation atténuée par rapport au volontaire sain. L'atteinte fibrosante pourrait altérer le passage transdermique de la molécule administrée. Néanmoins, d'autres facteurs pourraient également être impliqués. Les essais chez le rat de la iontophorèse de sitaxentan n'avaient pas montré de résultats concluants. Les iontophorèses de sitaxentan chez le rat après injection intra artérielle d'endothéline se rapprochant d'une situation pathologie ont montré une vasodilatation (*Roustit et al. Soumis*). L'argument que les molécules que nous avons sélectionnées sont toutes des molécules ayant montré leur efficacité dans une atteinte vasculaire dans la ScS apparaît comme un argument fort pour envisager un résultat positif de la iontophorèse thérapeutique avec le tréprostinil.

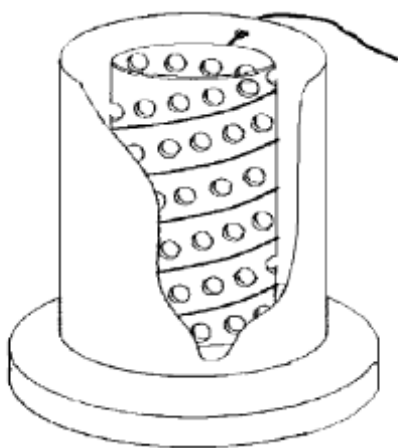
4/ La iontophorèse thérapeutique sera-t-elle concrètement réalisable chez le patient ScS ?

Les atteintes du patient ScS sont préférentiellement acrales. Un traitement par iontophorèse thérapeutique devrait répondre à deux contraintes : une surface suffisante traitée et un dispositif le plus conformable possible à la rétraction cutanée de la main sclérodermique. Les protocoles INFLUX-VS et INFLUX IT-VS ont été réalisés avec des sondes de iontophorèse

permettant une surface circulaire d'application de la molécule sélectionnée de 1,2 cm² (soit 360 µl de solution). Une telle surface à traitée est trop réduite pour être proposée dans une application thérapeutique chez le patient ScS.

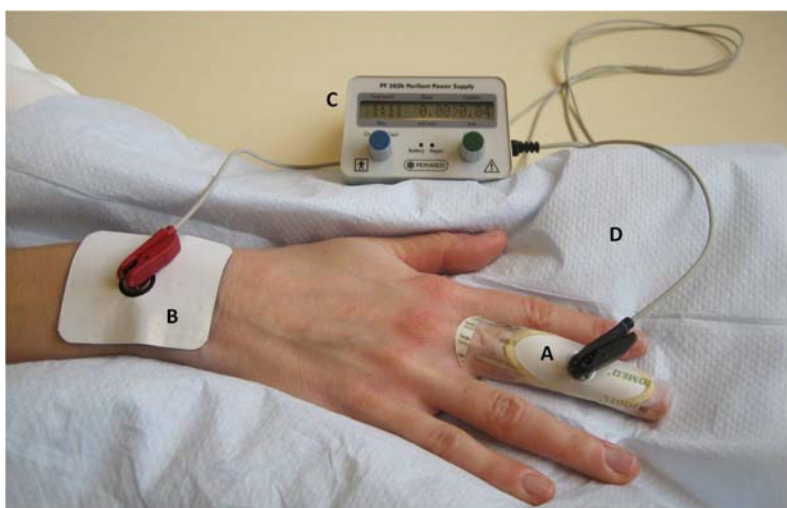
Murray, la première, avait pensé à un boîtier permettant le traitement par iontophorèse de l'ensemble d'un doigt, boîtier rigide dont on comprend aisément les limites à son application pour une main de patient ScS (*figure 25*), mais qui a le mérite de progresser dans le concept de iontophorèse thérapeutique avec l'objectif de traiter au moins un doigt, même si le dispositif n'est pas compatible avec l'évolution possible d'une sclérodactylie telle qu'une main « en griffe » .

Figure 25 : chambre de iontophorèse proposée par Murray (dessin schématique à gauche et concrète à droite)(Murray et al., 2005)



La technologie évolue et le protocole TIPPS va être réalisé avec des sondes actives Iogel (Iomed Inc., Salt Lake City, UT, USA) ayant une surface de 7,2 cm² (*figure 26*).

Figure 26: Iontophorèse digitale avec le dispositif Iogel (photo réalisée au CIC de Grenoble). A) électrode active (cathode) de 7,2 cm². B) électrode passive (anode). C) générateur de courant continu. D) coussin à vide permettant d'immobiliser le bras.



Les évolutions technologiques pourront peut-être permettre à termes, en parallèle des études pharmacologiques permettant de prouver l'efficacité d'une molécule candidate à la iontophorèse, un mode de délivrance de la iontophorèse chez les patients ScS plus adapté.

Une des pistes de travail consisterait à développer un appareillage basé sur le principe des « tissus intelligents » qui pourraient être humidifiés de la solution avec le principe actif tout en étant conforme à la main.

5/ D'autres molécules sont-elles envisagées ?

La voie des PDE5A pourrait être une voie intéressante à tester en iontophorèse. Seul le sildénafil a pu être obtenu en poudre pour pouvoir être testé en iontophorèse chez le rat et nous nous sommes confronté à des problèmes de solubilisation. Il est récemment arrivé sur le marché du sildénafil soluble qui pourrait être testé mais dont les tests devraient être refaits au préalable chez l'animal.

Il existe d'autres analogues de la prostacycline tel que le béraprost qui pourraient être testés. Enfin, nous avons testé en iontophorèse chez le rat un calcium bloquant (nicardipine) sans résultat probant (données non publiées).

6/ D'autres études sont-elles envisagées pour une meilleure compréhension de la physiopathologie des patients ScS ?

D'autres études physiopathologiques dans la ScS permettront de mieux connaître la microcirculation cutanée chez le patient ScS et les outils d'étude de sa physiopathologie. L'étude SPECIES permettra notamment de mettre en place l'exploration de la microcirculation de la main chez le volontaire sain, les patients avec RP primaire et les patients sclérodermiques avec le LSCI. Il s'agit d'une étude pilote ayant pour objectif de comparer une imagerie à l'état de base et après une hyperhémie postocclusive sur la face dorsale et palmaire entre 3 groupes : un groupe de volontaires sains, un groupe de sujets présentant un phénomène de Raynaud primaire, un groupe de sujets sclérodermiques.

Les comparaisons se feront en étudiant l'hyperhémie post occlusive en étudiant les différentes zones spatiales de la main avec une analyse séparée chez le patient ScS qu'il présente ou non une UD. L'évolution des techniques d'administration en iontophorèse vont de pair avec l'évolution technologique des méthodes d'enregistrement du signal, tel que le montrent certains résultats d'études : certains auteurs ont conclu en une inefficacité du Bosentan sur l'augmentation du flux sanguin cutané en fluxmétrie (Hettema *et al.*, 2009) alors que le Bosentan aux mêmes posologies et pendant la même durée de 16 semaines augmente la perfusion sanguine cutanée au niveau des mains des patients ScS (Rosato *et al.*).

L'étude Species est donc une évaluation non invasive de la dysfonction microvasculaire qui répond à plusieurs besoins :

- celui d'un test d'évaluation précoce de la dysfonction microvasculaire de la sclérodermie. La plupart des patients sclérodermiques se présentent initialement avec un phénomène de Raynaud, qui précède la fibrose cutanée et/ou pulmonaire. La capillaroscopie unguéale et la recherche d'auto anticorps sont utilisés pour déterminer quels patients sont susceptibles de développer une sclérodermie. Il existe une place pour des tests fonctionnels, et nos données suggèrent que l'étude de la réponse hyperhémique locale post occlusive en LSCI pourrait permettre de distinguer les patients susceptibles de développer une sclérodermie parmi ceux présentant un phénomène de Raynaud primaire.
- celui d'un test d'évaluation fine intermédiaire de la réponse thérapeutique.
- celui d'un test pronostique de susceptibilité à la survenue d'évènement grave tel que les ulcérations digitales. En effet, l'excellente résolution spatiale couplée à une exploration dynamique pourrait permettre de détecter les zones hypoperfusées susceptibles d'être ulcérées avant que les lésions cutanées n'apparaissent.

En conclusion, au cours de mon travail de thèse, nous avons montré qu'il était possible de potentialiser la iontophorèse de nitroprussiate de sodium pour la prise orale de 100 mg de sildénafil, et que les analogues de la prostacycline sont de bon candidats à une iontophorèse thérapeutique chez le sujet sclérodermique. Nous avons également évalué la reproductibilité des différentes techniques de quantification du flux microvasculaire cutané. Ces travaux ouvrent de nombreuses perspectives dans le domaine de la détection et la prise en charge des troubles trophiques de la sclérodermie systémique.

INDEX

AMM : autorisation de mise sur le marché
AUC : aire sous la courbe
CGRP(calcitonine gene-related peptide)
CVC : conductance cutanée vasculaire
CV :coefficient de variation
ET-1 :endothéline-1
ETA : récepteurs de l'endothéline A
ETB : récepteurs de l'endothéline B
EPCs : cellules progénitrices endothéliales
FMD : dilation flux-médiée de l'artère brachiale
HTAP : hypertension artérielle pulmonaire
HPO : hyperhémie post occlusive
ICC : coefficient de corrélation intra-classe
LDF : Laser Doppler Fluxmétrie
LDI : Laser Doppler Imageur
LSCI : Laser Speckle Contrast Analysis
MMP12 : matrice metalloprotéinase 12
NYHA :New York Heart Association
NO : oxide nitrique
PDE5A : phosphodiesterase de type 5A
PDF: Platelet Derivated Growth Factor
PGI2 : prostacyclines
RCS : Raynaud's Condition Score
RP : phénomène de Raynaud
ScS : Sclérodermie systémique
SNP : nitruressiate de sodium
TGF β : Tumor Growth Factor β
TNF α : Tumor Necrosis Factor α
TNF β : Tumor Necrosis Factor β
UD : ulcérations digitales
VEGF : Vascular Endothelial Growth Factor
vSMCs: cellules musculaires lisses vasculaires

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