TREPROSTINIL IONTOPHORESIS IN IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION

by

ADRIANO ROBERTO TONELLI, MD

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We hereby approve the thesis/dissertation of

Adriano Tonelli MD

candidate for the degree of Master of Science.

Committee Chair
Raed Dweik MD
Committee Member
Wilson Tang MD
Committee Member
James Spilsbury PhD MPH

Date of Defense
March 10, 2015

*We also certify that written approval has been obtained
for any proprietary material contained therein.
Dedication:

To my family, mentors, collaborators and patients
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List of abbreviations:

Treprostinil Iontophoresis In Idiopathic Pulmonary Arterial Hypertension

Abstract

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Objective:
Abnormalities in the prostacyclin pathway are involved in pathobiology of pulmonary arterial hypertension (PAH) and can be studied noninvasively by cutaneous treprostinil iontophoresis.

Methods:
Treprostinil iontophoresis was tested in 24 patients with idiopathic or heritable PAH and 25 age- and gender-matched controls, between 2/2013 and 2/2015.

Results:
The percentage change in perfusion units (PUs) with treprostinil iontophoresis was significantly reduced in IPAH patients compared with matched controls (median (interquartile range) 246 (96-605) versus 538 (381-1,035), p=0.004). The peak PUs were associated with NYHA functional class (R=-0.35, p=0.01), number of PAH therapies (R=0.48, p=0.02) and cardiac index (R=0.41, p=0.05, respectively). Lower peak PUs related to increased oxidative stress and arginine methylation products in plasma.

Conclusion:
Patients with IPAH have a decrease in the cutaneous microvascular response to treprostinil iontophoresis compared with matched controls; a finding that can be due to increased oxidative stress and abnormalities of the nitric oxide pathway.
Introduction:

Pulmonary arterial hypertension (PAH) is a progressive disease defined by abnormally elevated pulmonary pressures that can lead to right heart failure and death \(^1\). PAH kills patients at a productive age and at a tremendous financial cost. One of the most common types of PAH is idiopathic PAH (IPAH), a condition that has no identifiable etiology and causes progressive narrowing of the pulmonary arteries \(^2\). Initial studies characterized IPAH as a disease of excessive vasoconstriction and vascular rarefaction of the pulmonary circulation; however, recent data from us \(^3,4\) and other investigators \(^5-7\) showed that this view is incomplete since there is evidence of extrapulmonary vascular involvement. This extrapulmonary microvascular compromise in IPAH could result from an augmented oxidative stress and/or increased plasma levels of arginine methylation products, since both are associated with endothelial dysfunction \(^8-10\).

It remains unclear if the skin microcirculation mirrors the pulmonary vascular dysfunction seen in IPAH \(^11\). However, in other diseases, a more severe dysfunction of the cutaneous microcirculation is associated with disease severity and higher cardiovascular mortality \(^12\). The study of the extrapulmonary microcirculation (vessels < 100 µm) in IPAH may provide a noninvasive and mechanistic way of assessing the pulmonary circulation. Currently there are limited tests that evaluate the pulmonary circulation and only one study that predicts the long-term response to calcium-channel blocker therapy in PAH patients. Indeed, the hemodynamic response to inhaled nitric oxide (NO), intravenous epoprostenol, intravenous adenosine or inhaled iloprost is currently used to determine whether an IPAH patient would respond to treatment with calcium channel blockers \(^13,14\). However, this evaluation requires a right heart
catheterization (RHC) and does not help in deciding whether a patient would respond to PAH-specific therapies.

The extrapulmonary microcirculation can be studied in vivo with cutaneous laser Doppler flowmetry\textsuperscript{15}, which is a methodology that offers an effective and reliable way to challenge the cutaneous microcirculation and assess its function. Indeed, laser Doppler flowmetry can measure dynamic changes in flow over a small area of skin, in response to vasoreactive stimuli. One vasoreactive stimuli is the cutaneous administration of prostacyclin (PGI\textsubscript{2}) using iontophoresis, a methodology commonly referred as “an injection without the needle” that allow the local administration of minute quantities of a drug. Thus, the PGI\textsubscript{2} pathway can be tested at the level of the cutaneous microcirculation with treprostinil iontophoresis. Treprostinil is a PGI\textsubscript{2} analogue with neutral PH and good stability at room temperature\textsuperscript{16}. More importantly, it has previously shown to cause a sustained increase in cutaneous blood flow when delivered by cathodal iontophoresis to the skin of rats\textsuperscript{17}, healthy volunteers\textsuperscript{18} and patients with systemic sclerosis\textsuperscript{19}.

Abnormalities in the PGI\textsubscript{2} pathway constitute one of the main mechanisms involved in the pathogenesis of IPAH. Prostacyclin is a powerful vasodilator produced by the vascular endothelium that inhibits platelet adhesion and cell growth\textsuperscript{20,21}. It acts by interacting with the PGI\textsubscript{2} receptor and increasing cyclic adenosine monophosphate. Patients with IPAH have marked reduction in PGI\textsubscript{2} synthase in the lungs\textsuperscript{22} and lower plasma level and urinary excretion of PGI\textsubscript{2} metabolites\textsuperscript{23-25}. More importantly, PGI\textsubscript{2} analogues are effective therapies for treating IPAH\textsuperscript{26-28}.

We propose that the cutaneous microcirculation is a useful model for examining the underlying mechanisms responsible for the pulmonary vasculopathy of IPAH\textsuperscript{15,29}. 

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We hypothesize that in IPAH patients: a) the abnormalities in the PGI$_2$ pathway are systemic and can be studied in-vivo by cutaneous treprostinil iontophoresis, b) the increased oxidative stress and alteration in the NO pathway (higher levels of arginine methylation products, i.e. N-monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA)) affect the response to treprostinil iontophoresis in the cutaneous microcirculation.

**Methods:**

a) **Subjects and study design:**

The research protocol was approved by the Cleveland Clinic Institutional Review Board (study # 11-441) and all subjects provided written informed consent. The Division of Cardiovascular and Renal products of the Food and Drug Administration (FDA) concluded on June 15, 2012 that the present investigation met all the requirements for exemption from the Investigational New Drug Application (IND). We conducted this cross-sectional study between February 2013 and February 2015. We included consecutive patients that met hemodynamic criteria for PAH (defined as mean pulmonary artery pressure (PAP) $\geq$ 25 mm Hg, pulmonary artery wed pressure (PAWP) $\leq$ 15 mm Hg and pulmonary vascular resistance (PVR) $> 3$ Wood Units) and had the idiopathic (IPAH) or heritable (HPAH) forms of the disease. Four patients refused to participate, 3 because of time limitations and 1 due to lack of interest (n=1). We performed a detailed clinical evaluation in all patients to determine the etiology of PH. In addition, we studied age- and gender-matched controls subjects without evidence of pulmonary hypertension. Controls subjects were Cleveland Clinic employees that were recruited with the use of flyers.
Patients underwent RHC in the outpatient setting using local anesthesia (lidocaine 2%). No intravenous sedation or analgesia was given to any patient before, during or after the procedure. Patients did not eat or drink for 8 hours prior to testing. No patient or control was a current smoker. We continued or started O₂ supplementation in patients on chronic O₂ therapy or in whom the pulse oxygen saturation was below 90%, respectively. In the event that O₂ supplementation was needed, we maintained the same O₂ flow during all the study measurements. The procedure room had a stable temperature (72° F or 22° C) during all the interventions and patients and controls subjects were acclimatized to this temperature for at least 30 minutes.

b) Treprostinil iontophoresis:

We tested the effects of treprostinil iontophoresis and local thermal hyperemia with the PeriFlux system 5000 (Perimed, Järfälla, Sweden). This system uses Laser Doppler flowmetry which is based on the change in the wavelength of light (Doppler shift) when it hits moving blood cells. The magnitude of the change in wavelength is related to the number and velocity of the blood vessels. A linear relationship between laser Doppler signal and microvascular flow has been previously demonstrated. However, laser Doppler flowmetry does not provide an exact measure of flow (mL/min), in part because it is not possible to adequately determine the volume of tissue included in the measurements, therefore this methodology is predominantly used to test the microvascular reactivity. Measures are expressed as arbitrary perfusion units (PUs) where 1 PU represents 10 mV.

All studies were performed within 1 hour after the RHC. Probes were fully calibrated before each measurement. Probes sampled approximately 1 mm³ of tissue, a
volume that contains at least 50 microvessels, including arterioles, capillaries and venules.

Iontophoresis was performed using the thermostatic probe PF 481 and the perilont micropharmacology system (Perimed, Järfälla, Sweden) which allow the local administration of small volumes of vasoactive drugs. We used the drug delivery PF 383 and dispersive electrodes PF 384. Drug delivery electrodes are made of small sponge that is in direct contact with the skin and has a sideport for the electrical current and a central opening for the laser beam. Skin resistance and temperature were monitored throughout the study in all subjects. Treprostinil was delivered using an established protocol and great care was taken to create similar experimental conditions to ensure regional and temporal reproducibility. We measured local thermal hyperemia with an integrating probe (PF 413) to minimize variability of the determinations. Local thermal hyperemia was performed at 44° C for 20 minutes, using a probe holder as the heating element. This technique provides an index of neurovascular and NO-dependent blood flow regulation. The PU plateau, seen at 20 minutes (late plateau), is mostly NO-dependent. Data from the instrument were recorded continuously during all the studies for off-line review using the PeriSoft for Windows software. Probes were cleaned after measurements using a lint-free cloth moistened with alcohol. Careful attention was paid when placing the probes to not exert any pressure on the skin.

Patients were tested in a sitting position with the forearm at the level of the heart. We prepared the skin site by carefully applying a clean adhesive tape and then cleaning the area with 70 % alcohol. This process removed part of the stratum corneum. We then applied the drug-delivery electrode on the volar aspect of the forearm, 5 cm distal to the antecubital fossa, away from visible veins or skin abrasion. We placed 180 µL of
Treprostinil (concentration 1 mg/mL) on the sponge of the delivery electrode which had a surface of 1 cm$^2$. The dispersive electrode, which received opposite polarity, was positioned in the volar aspect of the forearm, 15 cm away from the drug delivery electrode (Figure 1). These electrodes were then connected to a power source that delivers the desired current intensity and polarity (a drug with negative charge is placed under the negatively charged electrode so it can be repelled into the dermis) \(^{37}\). Current-induced vasodilation was prevented by limiting the current density to < 0.01 mA/cm$^2$ \(^{38}\) while adjusting the concentration of drugs and vehicles, to maintain the maximum drug effect.

Treprostinil (Remodulin® 1mg/mL) and its placebo (treprostinil buffer) were iontophoresed for 30 minutes at 20 µA with a negative polarity. The PH of the medication was 6. Each mL of Remodulin ® placebo contained 5.3 mg sodium chloride, 3 mg metacresol, 6.3 mg sodium citrate, and water for injection. Sodium hydroxide and hydrochloric acid were added during the manufacturing process to adjust the pH between 6.0 and 7.2 \(^{16}\). Vials of treprostinil and placebo were discarded after 30 days of the initial introduction. Both the Remodulin and its placebo (treprostinil buffer) were provided by United Therapeutics (United Therapeutics Corp., NC, USA).

c) Functional, echocardiographic and hemodynamic measurements:

We determined the severity of PAH by using NYHA (New York Heart Association) functional class, plasma NT-pro brain natriuretic peptide concentration, diffusing capacity of carbon monoxide (DLCO) by single-breath technique \(^{39}\), distance walked in the 6-minute \(^{40}\) and right ventricular (RV) size and function on echocardiography \(^{41}\). RV function was determined subjectively and by the tricuspid
annular plane systolic excursion (TAPSE); meanwhile, RV size was measured in the apical four-chamber view using the RV basal, middle cavity and longitudinal dimensions.

We assessed quality of life with the Cambridge Pulmonary Hypertension Outcome Review (CAMPHOR) questionnaire, which is a specific measure of health-related quality of life for pulmonary hypertension that focuses on the physical and psychological impact of PH. The questionnaire encompasses 3 scales (overall symptoms [25-items, scored 0-25, where a higher score reflects more symptoms], function [15-items, scored 0-30, where a higher score reflects worse functioning] and quality of life scales [25-items, scored 0-25, where a higher score corresponds to poor quality of life]). Importantly, this questionnaire has been validated in the United States.

We measured and calculated the following hemodynamic parameters during RHC: right atrial (RA) pressure, mean PAP, PAWP, transpulmonary gradient (TPG: mean PAP - PAWP), cardiac index (CI) and PVR. Pulmonary vasodilatory challenge was performed using inhaled NO at 40 ppm for 5 minutes. Nitric oxide (INOmax, INO Therapeutics LLC, Hampton, NJ, USA) was delivered either on room air (4 L/min) or using the same flow of O₂ in the patients receiving supplementation. A positive response was considered present when the mean PAP decreased at least 10 mm Hg to an absolute value ≤ 40 mm Hg.

d) Laboratory determinations:

We measured a PGI₂ metabolite in plasma, i.e. 6-Keto Prostaglandin F1α, which is a stable hydrolyzed product of the unstable PGI₂. Levels of 6-keto-prostaglandin F1α are directly related with levels of PGI₂ hence they are the indicator of choice to measure.
PGI$_2$ levels $^{45}$. We measured the levels of 6-Keto Prostaglandin F1$\alpha$ in plasma using Sep-Pak columns for extraction and an ELISA assay (Oxford Biomedical Research, Oxford, MI, USA) for quantification. We also determined the ratio of reduced (GSH) over oxidized glutathione (GSSG) using the GSH/GSSG microplate assay (Oxford Biomedical Research, Oxford, MI, USA). Stable isotope dilution liquid chromatography-tandem mass spectrometry was used for quantification of plasma analytes (MMA and ADMA) as previously described $^{46}$.

In 5 control subjects we measured treprostinil levels in plasma, to determine whether there was systemic absorption. As a positive control we included 1 patient with IPAH treated with intravenous treprostinil (50 ng/kg/min). After 20 minutes of iontophoresis, venous blood was obtained from the contralateral antecubital fossa. Blood was rapidly processed (centrifuged at 3,000 rpm for 3 minutes) and plasma frozen at -80°C in K2 ethylenediaminetetraacetic acid (EDTA) tubes. These tubes were shipped overnight to Tandem Labs (Durham, NC, USA) in an insulated container with dry ice. Tandem Labs was not aware of the presence of a positive control. Treprostinil determinations were obtained with ultra-performance liquid chromatography and a triple quadrupole tandem mass spectrometer detector operated in negative TurboIonSpray mode. Calibration standards from 0.0100 to 5.00 ng/mL were used to construct standard curves for treprostinil.

e) Overall approach:

We used treprostinil iontophoresis to evaluate the cutaneous microvascular response to a PG$\text{I}_2$. We assessed the reproducibility of treprostinil iontophoresis, in a subgroup of subjects, by sequentially testing both forearms. Safety of the methodology
was ascertained by recording local and/or systemic side effects in all subjects, and by measuring treprostinil levels in plasma in 5 individuals. To determine whether the cutaneous microvascular response to treprostinil iontophoresis has any impact on the severity of IPAH, we compared this measurement with clinical, laboratory, echocardiographic and hemodynamic tests that traditionally reflect the severity of disease. We then studied the potential reasons for the abnormal cutaneous microvascular response to treprostinil by measuring 6-Keto Prostaglandin F1α (a stable metabolite of PGI2), GSH/GSSG ratio (a marker of oxidative stress) and MMA and ADMA (arginine methylation products associated with microvascular dysfunction in IPAH).

f) **Statistical analysis:**

Continuous data are presented as mean ± standard deviation (SD) or median (interquartile range (IQR)) as appropriate. Categorical data are summarized as discrete values and percentages (n (%)). Baseline and peak values are provided by the Perimed software after averaging, in our case, 30 seconds of recording. Results of the treprostinil iontophoresis test are expressed as peak PUs or percentage of variation from baseline. Since local hyperthermia produces maximal vasodilation 47, peak PUs with treprostinil iontophoresis were also adjusted for this factor and presented as a percentage of maximal PUs with local hyperthermia. Maximal PUs were obtained during the late plateau that occurs at 20 minutes of local hyperthermia. Some authors suggest dividing the PUs by systemic arterial pressure to obtain the cutaneous vascular conductance (PU over mean arterial pressure) 18. However, since the systemic pressure did not vary during the 30 minute course of treprostinil iontophoresis, this ratio is not shown.
Independent continuous samples were compared using Wilcoxon-Mann-Whitney test. Paired continuous samples were tested with Wilcoxon signed-rank test. Categorical variables were compared with Fisher’s exact test. Relationships between parameters were tested using the Pearson correlation test. Binary logistic regression was used to determine whether the percentage change in PUs with treprostinil continued to be a significant predictor of the presence of PAH, when adjusted by the continuous covariate skin resistance. Receiver Operating Characteristic (ROC) curves were constructed and values provided as area under the curve (AUC) with their respective 95% confidence interval. The optimal cut-off point was determined using the Youden index\(^48\). All \(p\) values are two-tailed and a value of \(< 0.05\) was considered significant. The statistical analyses were performed using the statistical package IBM SPSS, version 20 (IBM; Armonk, New York) and MedCalc, version 14.12.0 (Ostend, Belgium).

**Results:**

a) **Patient characteristics:**

Patients with IPAH (n=24; IPAH=22, HPAH=2) and controls (n=25) had similar age and gender (Table 1). We matched conditions known to be associated with systemic microvascular dysfunction such as systemic hypertension, sleep apnea, obesity, hypothyroidism and hypercholesterolemia. Two IPAH patients had well-controlled type 2 diabetes, with normal blood glucose levels on metformin. Patients with IPAH were in NYHA functional class I (17%), II (29%), III (50%) and IV (4%). Hemodynamic determinations in IPAH were RA: 8.4 ± 6.4 mm Hg, mean PAP: 44.5 ± 14 mm Hg, PAWP: 11.2 ± 5.6 mm Hg, TPG: 33.3 ± 11.5 mm Hg, CI (thermodilution): 2.8 ± 0.79 L/min/m\(^2\), PVR: 7.5 ± 4.3 Wood units.
At total of 17 IPAH patients had inhaled NO challenge and experienced an average percentage decrease in mean PAP and PVR of 11.3 ± 12.3 % and 14.0 ± 22.6 %, respectively. Only 3 of these patients had a positive vasodilator response by current hemodynamic criteria. The CAMPHOR scores for symptoms, activities and quality of life were 12.3 ± 6.6, 11.3 ± 7.6 and 7.7 ± 5.6, respectively. Distance walked on 6-minute was 319 ± 148 meters. The RV function on echo was normal, mild, moderate and severe in 38, 19, 24 and 19 % of IPAH patients, respectively. The TAPSE was 2.0 ± 5.8 cm and the RV basal, mid cavity and longitudinal dimensions were 4.3 ± 0.8 cm, 3.6 ± 1.1 cm and 7.5 ± 1.5 cm, respectively.

Three quarters of IPAH patients were receiving PHA-specific therapies at the time of treprostinil iontophoresis. Of these, 6 (25%) were on 1 therapy, 7 (29%) on 2 and 5 (21%) on 3 PAH-specific medications. Nine (38%) patients were on endothelin receptor antagonists, 14 (58%) on phosphodiestearase-5 inhibitors, 5 (21%) on inhaled and 7 (29%) on parenteral PGI₂ analogues.

b) Treprostinil iontophoresis is reduced in IPAH patients

The percentage change in PUs with treprostinil iontophoresis was significantly reduced in IPAH patients when compared with matched controls (246 (96-605) % versus 538 (381-1,035) %, respectively, p=0.004) (Figure 2, panel A). The percentage of peak PUs relative to the maximum value obtained by local hyperthermia was also significantly lower in IPAH than controls (Table 2). This relationship persisted even after correcting for skin resistance. The ROC curve that tested the ability of percentage change in PUs with treprostinil iontophoresis to discriminate between IPAH and controls showed an AUC of 0.75 (95% CI: 0.60-0.86, p=0.0007) and an optimal cut-off value by Youden
index of $\leq 249\%$ (sensitivity and specificity of 54.2 and 91.3\%, respectively) (Figure 3, panel A).

To test the possibility that treprostinil iontophoresis is affected by treatment with PGI$_2$ analogues we repeated the analyses in the subgroup of IPAH patients not on PGI$_2$ analogues (n=12). In IPAH patients not treated with PGI$_2$ analogues the peak PUs (11 (7-23) versus 31 (18-66), p=0.001), percentage change in PUs (225 (86-274) % versus 538 (381-1035) %, p<0.001) (Figure 2, panel B) and % change in PUs relative to maximal dilation with local hyperthermia (7.5 (4.9-21.9) % versus 21.4 (11.5-41.1) %, p=0.02) with treprostinil iontophoresis were lower than matched control subjects (n=23). The ROC curve of percentage change in PUs showed an AUC for discriminating IPAH versus matched controls of 0.87 (95% CI: 0.72-0.96, p<0.0001) with a cut-off value by Youden index of $\leq 300\%$ (sensitivity of 83.3 % and specificity of 87.0 %) (Figure 3, panel B).

c) **Validity of the measurements during treprostinil iontophoresis:**

In 6 subjects (2 IPAH patients and 4 controls), we sequentially iontophorese treprostinil and the placebo of Remodulin®, on slightly different sites. We found that the peak PUs (32.5 (22.5-51.0) versus 8.9 (4.8-12.6), p=0.03) and percentage change in PU (354.5 (257.1-743.4) % versus 45.1 (0-121.3) %, p=0.03) were higher in those individuals receiving treprostinil, supporting that the vasoactive effects are mostly due to treprostinil and not the placebo components of Remodulin®. In 5 patients, we performed treprostinil iontophoresis in both forearms. In these subjects the percentage change in PUs was similar with an intraclass correlation coefficient for single measures of 0.86, p=0.008. Measures are independent of the observer as results are automatically generated by the PeriSoft for Windows software.
d) Relationship between treprostinil iontophoresis and functional, echocardiographic and hemodynamic determinations in IPAH.

The peak PUs during treprostinil iontophoresis had an association with NYHA functional class (R=-0.35, p=0.01) and the number of PAH-specific therapies (R=0.48, p=0.02). We also noted a significant association between peak PU and percentage change in PUs with treprostinil iontophoresis and CI measured by thermodilution (n=23, R=0.57, p=0.004 and R=0.41, p=0.05, respectively) or direct (measured) Fick methodology (n=9, R=0.83, p=0.006 and R=0.86, p=0.003, respectively). There were no significant associations between cutaneous microvascular determinations during treprostinil iontophoresis and plasma NT-pro brain natriuretic peptide, 6 minute walk distance, DLCO, RV function or dimensions, or changes in mean PAP or PVR during NO pulmonary vasoreactivity challenge.

e) Comparison of IPAH patients treated or not treated with PGI₂ analogues.

Twelve patients with IPAH were treated with PGI₂ analogues (IV: 7 [4 treprostinil and 3 epoprostenol] and inhaled: 5 [all inhaled treprostinil]). Patients treated with PGI₂ analogues had higher baseline PUs (7 (4-10) versus 3 (2-6), p=0.043) and higher CI by thermodilution (3.2 (2.8-3.6) L/min/m² versus 2.4 (1.8-3.1) L/min/m², p=0.013). In addition, those receiving PGI₂ analogues had a higher peak PUs (517 (202-672) versus 225 (86-274), p=0.004) and possibly higher percentage change in PUs (13.4 (6.7-33.0) versus 7.5 (4.9-21.9), p=0.06) during treprostinil iontophoresis when compared with individuals not receiving this treatment.
Interestingly, in this group of IPAH the level of 6-Keto Prostaglandin F1α (n=6), showed an inverse association with mean PAP (R=-0.81, p=0.05) and TPG (R=-0.88, p=0.02). In this limited sample, the plasma level of prostaglandin F1α was not significantly associated with % change in PUs with treprostinil iontophoresis (R=-0.34, p=0.51).

f) Impact of oxidative stress and arginine methylation products on the response to treprostinil iontophoresis:

In 12 subjects, an increased level of oxidative stress as measured by GSH/GSSG (the lower the ratio, the higher the oxidative stress) was associated with a decrease in peak PUs with treprostinil iontophoresis (R=0.63, p=0.03). In ten IPAH patients we also measured MMA and ADMA and noted that the percentage increase in PUs was associated MMA (R=-0.86, p=0.002) and ADMA (R=-0.71, p=0.02) plasma levels.

g) Effects of treprostinil iontophoresis:

Only minor side effects were noted during treprostinil iontophoresis including mild temporary itching and local erythema of the skin that resolved in a few hours. No skin damage (irritation, edema, burns, etc), pain or systemic side effects were observed. Furthermore, in 6 subjects we tested the plasma concentration of treprostinil after 20 minutes of iontophoresis. We confirmed that the treprostinil concentration in all 5 plasma samples of control subjects was lower than the quantification threshold (10 pg/mL). The IPAH patient on IV treprostinil had a determination above the treprostinil determination threshold (5.00 ng/mL).
Discussion:

Prostacyclin is a major vasodilator produced in the endothelial cells from prostaglandin H\(_2\) by the enzyme prostacyclin synthase\(^{20,21}\). The PGI\(_2\) pathway is important in the pathobiology of PAH. In fact, the enzyme prostacyclin synthase is reduced in the lung and the PGI\(_2\) metabolite, i.e. 6-Keto Prostaglandin F1\(\alpha\), is decreased in plasma and urine of IPAH patients\(^{22,23,24}\). We now showed that the cutaneous microvasculature of IPAH patients has a decreased vasodilatory response when challenged with treprostinil delivered by iontophoresis. This reduced vasodilatory response was more pronounced in IPAH patients who were not receiving PGI\(_2\) analogues. An increased oxidative stress and alteration in the NO pathway may in part explain the decrease response to treprostinil iontophoresis in patients with IPAH.

Iontophoresis is a methodology that uses direct electrical current to enhance the absorption of drugs into a patch of skin\(^{49,50}\). This technique makes possible to study the effect of vasoactive drugs such as treprostinil on the cutaneous microvascular bed. Blaise et al. challenged the cutaneous microcirculation with treprostinil iontophoresis in healthy volunteers and measured the response with laser Doppler imaging\(^{18}\). The authors showed that 20 minutes of 20 \(\mu\)A of continuous cathodal iontophoresis of treprostinil (concentration 0.1 mg/mL after dilution of the 1mg/mL solution with NaCl 0.9%) was well tolerated and induced a large and sustained cutaneous vasodilation with a peak at 30 minutes. Also in healthy volunteers, Roustit et al. showed a peak in PUs, measured with laser speckle contrast imaging, at 30 minutes of the initiation of treprostinil iontophoresis. Furthermore, Roustit et al. showed that the peak dermal concentration of treprostinil was achieved at 2 hours and its level was detectable for up to 8 hours after the end of
iontophoresis. Meanwhile plasma treprostinil was not detected in these individuals. Our protocol used the same current intensity but a higher concentration of treprostinil (1 mg/mL) and a longer duration of administration (30 minutes). We used a higher treprostinil concentration to be able to apply the medication out of the bottle without dilutions. The longer protocol duration was made to coincide with the peak of flux noted by previous studies. We showed that the vasodilatory effects noted were mostly due to the active agent and not the Remodulin® diluent or the small increase in the temperature of the delivery electrode.

We have previously presented that in patients with PAH, the sublingual vessels have a reduced flow with greater heterogeneity and more pronounced tortuosity than age- and gender-matched healthy controls. Similarly, other authors found a lower capillary density with nailfold capillaroscopy and a loss of the skeletal muscle microcirculation in IPAH patients. We noted that the transcutaneous oxygen (O₂) levels, measured non-invasively at the level of the forearm, are reduced in patients with PAH compared with age- and gender-matched healthy controls. This reduction in transcutaneous O₂ likely reflects a decrease in peripheral perfusion and cutaneous capillary density.

Collectively these results suggest that IPAH is a global vasculopathy with a primary manifestation in the lungs. It remains unclear whether the pulmonary vascular disease occurs first and then affects the systemic vasculature or the same trigger simultaneously involves both circulations with a predominantly pulmonary vascular manifestation.

We now present further evidence that the cutaneous microcirculation at the level of the forearm is affected in IPAH compared with matched controls. A potential
explanation for this finding includes a decrease in the endothelial synthesis of PGI2 in IPAH patients, which results in a decrease in the skin capillary density and less response to treprostinil iontophoresis (Figure 4). A constricted microvascular bed decreases the transcutaneous drug delivery with iontophoresis. In fact, patients with IPAH receiving PGI2 analogues had a higher baseline and peak PUs with treprostinil iontophoresis.

Actually it is common to observe that patients who received PGI2 analogues develop skin erythema / flushing. This side effect could be due to an increase in the CI (as seen in our study) and / or skin capillary density which leads to better skin perfusion (supported by higher baseline PUs seen in IPAH patients on PGI2 analogues) and more pronounced response to treprostinil iontophoresis. Interestingly, the percentage change in PUs with treprostinil iontophoresis showed good sensitivity and specificity for discriminating IPAH from matched controls particularly in IPAH not receiving PGI2 analogues.

Other reasons for a decrease in the cutaneous microvascular response to treprostinil iontophoresis (Figure 4) include an accelerated metabolism of PGI2 in IPAH patients which is suggested by a higher level of PGI2 metabolites in IPAH patients. An alternative explanation includes the increased oxidative state observed in IPAH patients. Indeed, we observed that a lower GSH/GSSG ratio (the lower the ratio, the higher the oxidative stress) was associated with a decreased peak PUs (R=0.63, p=0.03). In addition, higher levels of MMA and ADMA were associated with lower vasodilatory response to treprostinil iontophoresis. Interestingly, the oxidative stress can affect both the nitric oxide and PGI2 pathways resulting in a diminished capacity to synthetize these vasodilators. For instance, peroxynitrite may cause endothelial NO synthase uncoupling and adversely affect the activity of prostacyclin synthase by endothelial nitric
oxide synthase dependent tyrosine nitration\textsuperscript{57,58}. In support of this, Gabrielli et al. showed that the acute pulmonary vascular response to inhaled PGI\textsubscript{2} analogues is lower in IPAH with a higher oxidative stress and endothelial dysfunction\textsuperscript{8}.

There is an increased oxidative stress in PAH\textsuperscript{59-69} that may have severe implications for the overall cardiovascular health\textsuperscript{32}. The increased oxidative state plays an essential role in the pathobiology of PAH since reactive oxygen species participate in pulmonary vascular remodeling, microvascular dysfunction, and disease progression\textsuperscript{8}. Reactive oxygen species can inhibit the PGI\textsubscript{2} and NO endothelium-dependent vasodilator pathways. The is evidence of the constant cross talk between NO and PGI\textsubscript{2} at many levels, but having its central feature in the modulation of molecular mechanisms that regulate the PGI\textsubscript{2} pathway\textsuperscript{70}. In fact, with oxidative stress comes a shift in the metabolism of arachidonic acid from predominant production of PGI\textsubscript{2} to the vasoconstrictor thromboxane A\textsubscript{2}\textsuperscript{71}.

Lungs generate a significant amount of ADMA that contributes to tissue and plasma ADMA levels\textsuperscript{10}. Asymmetric dimethylarginine (ADMA) is produced by endothelial cells and inhibits endothelial NO synthase by competing with L-arginine for active site of the enzyme. ADMA is a critical regulator of vascular endothelial function and elevated levels are associated with more severe endothelial dysfunction and PAH\textsuperscript{9,10}. Moreover, elevated levels of ADMA in PAH are associated with worse survival\textsuperscript{9,69,72 69}.

In our study, the response to treprostinil iontophoresis was directly associated with the CI, a determination that was higher in IPAH individuals treated with PGI\textsubscript{2} analogues. Conversely, this association was not maintained in the subgroup of IPAH patients not treated with PGI\textsubscript{2} analogues. It is possible that PGI\textsubscript{2} analogues increased the
cardiac index and the cutaneous capillary density by recruitment / vasodilation and this effect allowed a higher response to treprostinil iontophoresis.

Our study has limitations: 1) the number of PAH is relatively small given that we only included IPAH or HPAH because other types of PAH are associated with diseases that can affect the systemic microcirculation, e.g. connective tissue diseases, cirrhosis with portal hypertension, etc. 2) most of the IPAH patients studied were on PAH-specific medications, reflecting the existing difficulties in identifying treatment naïve IPAH patients in referral centers. 3) we did not find an association between the pulmonary vascular response to inhaled NO and the percentage change en PUs with treprostinil iontophoresis potentially because three quarters of IPAH patients were on PAH-specific therapies and only 3 had a positive response. In contrast, Wolff et al. found a strong association between peripheral microvascular function measured by flow-mediated vasodilation and percent decrease in mean PAP and PVR after iloprost 73 which supported the relationship between systemic microvascular and pulmonary vascular response. Similarly, Gabrielli et al. showed that impaired flow mediated dilation is affected in PAH possibly because of an increased oxidative stress that leads to a decreased response to inhaled PGI2 analogues 8. These 2 studies are not comparable to ours because we challenged the pulmonary vasculature and systemic microcirculation with inhaled NO and treprostinil iontophoresis, respectively.

Notwithstanding these limitations, our research supports that the cutaneous microvasculature is affected in IPAH and possibly improves with PGI2 analogues treatment. Several factors gravitate in the decreased response of the cutaneous microvasculature to treprostinil iontophoresis including increased oxidative stress and
defects in the NO pathway. More importantly, our study shows that it is possible to test the PGI₂ pathway in vivo in patients with IPAH and this approach may help phenotype IPAH patients based on the cutaneous microvascular response to vasoactive agents. Further research would be needed to determine if treprostinil iontophoresis can help guide treatment decisions that change relevant outcomes. We are currently performing treprostinil iontophoresis in patients before and after 3 months of PGI₂ analogue therapy, to determine whether the initial (PUs percentage change) or degree of variation (difference between PUs percentage change at baseline and after 3 months) in cutaneous microvascular response could predict response to this group of PAH-specific treatments.

**Conclusions:**

Patients with IPAH have a decrease in the cutaneous microvascular response to treprostinil iontophoresis when compared with matched controls; a finding that can be due to increased oxidative stress and alteration in the nitric oxide pathway. Treatment with PGI₂ analogues is associated with a more pronounced microvascular response to treprostinil iontophoresis.
Table 1: Baseline characteristics of IPAH patients and controls

<table>
<thead>
<tr>
<th></th>
<th>IPAH Mean ± SD / n (%)</th>
<th>Controls Mean ± SD / n (%)</th>
<th>P (Wilcoxon and Fisher’s exact test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>24</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.4 ± 15.0</td>
<td>51.6 ± 8.7</td>
<td>0.40</td>
</tr>
<tr>
<td>Female gender</td>
<td>20 (83)</td>
<td>18 (72)</td>
<td>0.50</td>
</tr>
<tr>
<td>Systemic hypertension*</td>
<td>7 (29)</td>
<td>7 (28)</td>
<td>1</td>
</tr>
<tr>
<td>Sleep apnea*</td>
<td>5 (21)</td>
<td>5 (20)</td>
<td>1</td>
</tr>
<tr>
<td>Obesity</td>
<td>4 (17)</td>
<td>3 (12)</td>
<td>0.70</td>
</tr>
<tr>
<td>Hypothyroidism*</td>
<td>1 (4)</td>
<td>2 (8)</td>
<td>1</td>
</tr>
<tr>
<td>Hypercholesterolemia*</td>
<td>3 (13)</td>
<td>3 (12)</td>
<td>1</td>
</tr>
<tr>
<td>Type II diabetes *</td>
<td>2 (8)</td>
<td>0 (0)</td>
<td>0.23</td>
</tr>
</tbody>
</table>

* These conditions were adequately treated in IPAH patients and controls. The 2 patients with diabetes had their blood sugar normalized on metformin.

**Abbreviations:** IPAH: idiopathic pulmonary arterial hypertension, IQR: interquartile range, SD: standard deviation.
Table 2: Results of treprostinil iontophoresis in IPAH patients and controls

<table>
<thead>
<tr>
<th>All IPAH patients</th>
<th>IPAH median (IQR) / n (%)</th>
<th>Controls median (IQR) / n (%)</th>
<th>P (Wilcoxon test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>24</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Baseline PUs</td>
<td>5 (3-9)</td>
<td>5 (3-6)</td>
<td>0.69</td>
</tr>
<tr>
<td>Skin temperature at baseline (°C)</td>
<td>26.8 (25.5-27.6)</td>
<td>26.8 (25.8-27.7)</td>
<td>0.61</td>
</tr>
<tr>
<td>Skin temperature at 20 minutes of iontophoresis (°C)</td>
<td>29 (27-31)</td>
<td>30 (29-31)</td>
<td>0.70</td>
</tr>
<tr>
<td>Skin resistance (mOhms)</td>
<td>272 (199-456)</td>
<td>359 (286-445)</td>
<td>0.48</td>
</tr>
<tr>
<td>Voltage (mV)</td>
<td>5.4 (4.0-9.0)</td>
<td>7.1 (5.7-8.8)</td>
<td>0.48</td>
</tr>
<tr>
<td>Peak PUs with treprostinil iontophoresis</td>
<td>21 (10-37)</td>
<td>31 (18-66)</td>
<td>0.05</td>
</tr>
<tr>
<td>Change in PUs with treprostinil iontophoresis (%)</td>
<td>246 (96-605)</td>
<td>538 (381-1035)</td>
<td>0.004</td>
</tr>
<tr>
<td>Change in PUs with local hyperthermia (%)</td>
<td>1497 (858-1858)</td>
<td>1393 (994-1742)</td>
<td>0.96</td>
</tr>
<tr>
<td>Maximal PUs as a percentage of peak PUs with local hyperthermia (%)</td>
<td>11.7 (5.8-27.3)</td>
<td>21.4 (11.5-41.1)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Abbreviations: IPAH: idiopathic pulmonary arterial hypertension, IQR: interquartile range, PUs: perfusion units, SD: standard deviation.
Table 3: Results of treprostinil iontophoresis in IPAH patients not on PGI$_2$ analogues and controls.

<table>
<thead>
<tr>
<th></th>
<th>IPAH median (IQR) / n (%)</th>
<th>Controls median (IQR) / n (%)</th>
<th>P (Wilcoxon test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>12</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Baseline PUs</td>
<td>3 (2-6)</td>
<td>5 (3-6)</td>
<td>0.21</td>
</tr>
<tr>
<td>Skin temperature at baseline (°C)</td>
<td>26.9 (26.7-28.1)</td>
<td>26.8 (25.8-27.7)</td>
<td>0.61</td>
</tr>
<tr>
<td>Skin temperature at 20 minutes of iontophoresis (°C)</td>
<td>29 (27-32)</td>
<td>30 (29-31)</td>
<td>0.79</td>
</tr>
<tr>
<td>Skin resistance (mOhms)</td>
<td>332 (196-470)</td>
<td>359 (286-445)</td>
<td>0.85</td>
</tr>
<tr>
<td>Voltage (mV)</td>
<td>6.6 (3.9-9.3)</td>
<td>7.1 (5.7-8.8)</td>
<td>0.85</td>
</tr>
<tr>
<td>Peak PUs with treprostinil iontophoresis</td>
<td>11 (7-23)</td>
<td>31 (18-66)</td>
<td>0.001</td>
</tr>
<tr>
<td>Change in PUs with treprostinil iontophoresis (%)</td>
<td>225 (86-274)</td>
<td>538 (381-1035)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Change in PUs with local hyperthermia (%)</td>
<td>1579 (732-1735)</td>
<td>1393 (994-1742)</td>
<td>0.75</td>
</tr>
<tr>
<td>Maximal PUs as a percentage of peak PUs with local hyperthermia (%)</td>
<td>7.5 (4.9-21.9)</td>
<td>21.4 (11.5-41.1)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Abbreviations:** IPAH: idiopathic pulmonary arterial hypertension, IQR: interquartile range, PUs: perfusion units, SD: standard deviation.
Figure 1: Cutaneous Laser Doppler flowmetry and treprostinil iontophoresis.

Periflux system 5000 with the periIont micropharmacology system. Drug delivery electrodes are made of small sponge for the active drug and a sideport for the electrical application. Flow is measured in PU at the center of the electrode. **Inset figure:** delivery electrode placed on the skin. In this example, the electrode has (+) polarity to match the charge of the vasoactive agent (like charges repel each other). Perimed gave permission to publish this figure.
Figure 2: Boxplot of the percentage change in PUs with treprostinil iontophoresis in IPAH patients and controls.

Data are presented as Box-and-Whisker plots. Panel A compares all the IPAH versus controls. The horizontal line marks the 249 % change in PUs suggested by the Youden index. Panel B contrasts only the IPAH not receiving PGI₂ analogues against controls. The horizontal line marks the 300 % change in PUs obtained with the Youden index.
Figure 3: ROC curve in all IPAH patients (panel A) and those not receiving PGI$_2$ analogues (panel B).

The arrows mark the Youden index (Panel A: $\leq 249\%$ change in PUs and Panel B: $\leq 300\%$ change in PUs)
Figure 4: Mechanisms likely involved in the lower response to treprostinil iontophoresis in IPAH patients.

**Abbreviations:** ADMA: asymmetric dimethylarginine, MMA: N-monomethylarginine, NO: nitric oxide, PIG$_2$: prostacyclin, ROS: reactive oxygen species
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