Dysregulated expression of hypoxia-inducible factors augments myofibroblasts differentiation in idiopathic pulmonary fibrosis

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Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is an age-related, progressive and lethal disease, whose pathogenesis is associated with fibroblasts/myofibroblasts foci that produce excessive extracellular matrix accumulation in lung parenchyma. Hypoxia has been described as a determinant factor in its development and progression. However, the role of distinct members of this pathway is not completely described.

Methods: By western blot, quantitative PCR, Immunohistochemistry and Immunocitochemistry were evaluated, the expression HIF alpha subunit isoforms 1, 2 & 3 as well, as their role in myofibroblast differentiation in lung tissue and fibroblast cell lines derived from IPF patients.

Results: Hypoxia signaling pathway was found very active in lungs and fibroblasts from IPF patients, as demonstrated by the abundance of alpha subunits 1 and 2, which further correlated with the increased expression of myofibroblast marker αSMA. In contrast, HIF-3α showed reduced expression associated with its promoter hypermethylation.

Conclusions: This study lends further support to the involvement of hypoxia in the pathogenesis of IPF, and poses HIF-3α expression as a potential negative regulator of these phenomena.

Keywords: Hypoxia inducible factors; αSMA, Lung fibroblasts, HIF-1α, HIF-2α, HIF-3α, Methylation
Several studies have demonstrated a direct link between hypoxia and the development of IPF, principally by the notion that hypoxia worsens by constant deposition of ECM and vice versa [6–8]. Whole lung transcriptome of IPF patients has shown that hypoxia signaling pathway is upregulated and denotes the signature of HIF-1α [5, 6]. At cellular level hypoxia signaling by HIF-1alpha is able to modify the behavior of IPF derived fibroblasts increasing their proliferation [9, 10]. Therefore, the scarring of lung tissue in IPF is reinforced by the expansion of fibroblast/myofibroblasts foci. However, the correlation between fibroblast foci formation and hypoxia signaling is not well described, and we can now provide additional insights into this process.

Methods

Human lung fibroblasts

The study was approved by the ethics committee of the Instituto Nacional de Enfermedades Respiratorias (INER). Written informed consent was obtained from all participating individuals. Human fibroblasts cell lines were obtained by surgery (n = 4) and in the case of controls were taken from heathy lungs (n = 4) that were not suitable for transplant. IPF diagnosis depends on the department of interstitial lung diseases of the INER according to the ATS/ERS/ALAT guidelines [11, 12]. Approximately 20% of patients requiring surgery biopsy for definitive diagnostic, and this decision is established in an MDD (multi-disciplinary board) when tomographic criteria or clinical data are unclear. So this group is used in this study once the final diagnosis for IPF is confirmed (90% confidence). Fibroblasts were derived from lung tissue obtained from IPF patients and controls were isolated by enzymatic dispersion with trypsin (Sigma-Aldrich). Cells were grown with Ham’s F-12 medium (Gibco) supplemented with 100 U/mL of penicillin, 100 μg/ml of streptomycin, 2.5 mg/ml of amphotericin B and 10% FBS (Gibco); at 37°C; in an atmosphere of 95% air and 5% CO2; until reaching early confluence. All experiments were performed on cells with passage number between 5 to 10.

Hypoxia

For studying hypoxic conditions, fibroblast culture plates were transferred and maintained in a modular incubator chamber (Billups-Rothenberg Inc. CA.), at 37°C, for different periods of time, according to the experiment, in a humidified atmosphere with the following hypoxic gas mixture: 1% O2, 5% CO2, and balanced with N2. An Oxygen Analyzer (Teledyne Electronic Technologies 60 T) with an Oxygen Sensor (OOM105 of EnviteC-Wismar GmbH) to regulate the atmosphere composition.

Western blot

Cells were lysed with RIPA buffer (Sigma- Aldrich, St. Louis, MO). Protein quantification was performed using the BCA Protein Assay Kit (Thermo Scientific, USA). 30 μg from total extracts were separated by 10% SDS polyacrylamide gels (PAGE), and proteins were then transferred to nitrocellulose membranes. After nonspecific sites blockaded with 4% (wt/vol) of non-fat dried milk in phosphate-buffered saline (PBS), the membrane was incubated overnight with the correspondent antibody: HIF-1α (Abcam; ab1), HIF-2α (Novus; NB100–122) and HIF-3α (Novus; NBP1–03155), and αSMA (Sigma; A2547). β-actin (Sigma, A5441) was used as housekeeping. Bands were detected using secondary antibodies (Licor) and Odyssey scanner (Licor) detection system. Quantification was performed using ImageJ software (NIH).

Quantitative PCR

RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Grand Island, NY). One microgram of total RNA was used to generate cDNA (Advantage RT-for-PCR Kit; Clontech, Palo Alto, CA). Real-time PCR was carried out on a StepOne Thermocycler (Applied Biosystems, Foster City, CA) using Applied Biosystems TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA.) according to the manufacturer’s protocol. The expression assay was carried out using the following TaqMan probes: Hs00153153_m1 for HIF-1α, Hs01026149_m1 for HIF-2α (EPAS1), Hs00541709_m1 for HIF-3α, Hs00426835_g1 for ACTA2 (αSMA) all of the above labeled with FAM and normalized with Hs99999901_s1 for 18S ribosomal RNA labeled with VIC (Applied Biosystems, Foster City, CA). Relative quantitation method was used to analyze the results of two independent experiments made in triplicate. For each experimental sample, a gene was considered as not expressed if amplification was not detected by threshold cycle Ct = 40.

Immunohistochemistry on tissue microarrays and Immunocitochemistry

For lung samples tissue analysis, tissue microarrays (TMA) were constructed using paraffin blocks from the pathology archive. Samples for IPF (n = 10), other inflammatory diseases (n = 5), from lung cancer patients (n = 2), and normal residual lung tissue from patients with cancer (n = 7). The tissue microarray was constructed using one core of 5 mm in diameter per sample. Positive control tissue from normal kidney and placenta were used for optimal antibody titration, before immunohistochemistry reactions were performed on tissue microarray or cellular culture slides. All slides were prepared by placing positive and negative control tissue...
on them. Primary antibody specifications are shown in Table 1. For TMA paraffin sections the reactions were performed manually as follows: 1) Slide deparaffinization at room temperature in successive incubations in xylene, absolute ethanol and 96° ethanol, 2) Antigen retrieval with citrate buffer (pH 6, pressure cooker) for 10 min at 95°c for HIF-1α, HIF-2α and αSMA; and with 1x proteinase K (Diagnostic Biosystems, Pleasanton, CA) for 20 min at room temperature for HIF-3α; 3) Peroxidase blockade, using a 3% solution of hydrogen peroxide in methanol, for 10 min at room temperature; 4) Primary antibody incubation overnight in a humidity chamber on them. Primary antibody specifications are shown in Table 1. For TMA paraffin sections the reactions were performed manually as follows: 1) Slide deparaffinization at room temperature in successive incubations in xylene, absolute ethanol and 96° ethanol, 2) Antigen retrieval with citrate buffer (pH 6, pressure cooker) for 10 min at 95°c for HIF-1α, HIF-2α and αSMA; and with 1x proteinase K (Diagnostic Biosystems, Pleasanton, CA) for 20 min at room temperature for HIF-3α; 3) Peroxidase blockade, using a 3% solution of hydrogen peroxide in methanol, for 10 min at room temperature; 4) Primary antibody incubation overnight in a humidity chamber according to Table 1 titers; and 5) Polymer-horseradish peroxidase based detection with diaminobenzidine as chromogen (MACH 4, Biocare Medical, Pacheco, CA). In between all steps, thorough washings were made with Tween 20 – PBS. All slides were counterstained with Harris Haematoxylin and mounted with non-aqueous medium. For immunocytochemical reactions on slide cell cultures of fibroblasts lines, the procedure was similar to TMA paraffin sections, just substituting the initial deparaffinization steps for a membrane permeabilization step through: incubation in 70% methanol for 1 h. TMA immunohistochemistry reactions for HIF-1α, HIF-2α and HIF-3α were evaluated by a pathologist in fibroblasts, lymphocytes, macrophages, plasma cells and alveolar epithelium. Percentage of positive cells and reaction intensity were registered as categorical data according to the next ranges: A) Percentage.- 0 for 0% of positive cells, 1 for <10% of positive cells, 2 for 10–50% of positive cells and 3 for >50% of positive cells; and B) Intensity.- 0 for negative reactions, I for light staining (barely perceptible under 10x objective, but well defined stain visible under 40x objective), II for moderate staining (well defined not strong stain under 10x objective), and III for strong staining (evident intense stain even under 4x objective).

Demethylation test (5-Aza)
IPF fibroblasts were seeded in a 6-well plate at 30% confluence in complete F12 medium and were treated with 5-Aza-2′-deoxycytidine (5-Aza) (A3656 Sigma) for 5 days, the medium was changed every 24 h. After the treatment with the inhibitor, the cells were lysed.

| Target  | Antibody features | Trademark | Titer |
|----------|------------------|-----------|-------|
| HIF1α    | Mouse monoclonal | Abcam (ab 1) | 1:50  |
| HIF2α    | Rabbit polyclonal | Novus (NB100-122) | 1:150 |
| HIF3α    | Rabbit polyclonal | Novus (NBP1-03155) | 1:150 |
| ACTA     | Mouse Monoclonal | Sigma-Aldrich (A2547) | 1:1000 |

Statistical analysis
Before each statistical analysis of quantitative variables, a Shapiro-Wilk test was performed to evaluate normality distribution of the data. For western blot and genic expression analysis, the paired t-test was used for intra-group comparisons of each sample or group studied. Inter-group comparisons were performed using the Levene’s test and independent t-tests, Tukey tests, to investigate the differences between groups. All statistical analyses were performed with the IBM SPSS Statistics 20.0 (Chicago, Illinois, USA) software. P < 0.05 was considered statistically significant. For immunohistochemistry, results were compared between normal control tissue samples with either idiopathic pulmonary fibrosis or other pulmonary inflammatory conditions. 4 × 2 contingency tables were constructed, and the Freeman-Halton extension of the Fisher exact probability test performed on them.

Results
Hypoxia contributes to myofibroblast differentiation in IPF
αSMA expression was evaluated as a marker of myofibroblast differentiation which is a key feature in the pathogenesis of IPF. To determine expression levels of αSMA, four lines of fibroblasts controls were obtained with an average age similar to the lines derived from patients with IPF, all of them were exposed for 48 h to 1% Oxygen. Control fibroblasts showed a significant increase in the protein expression after hypoxia (p = 0.01) (Fig. 2 a-b). As it can be also see by immunocytochemistry in (Fig. 1d). IPF derived fibroblasts exhibited a significant difference in baseline conditions compared to controls (p = 0.008); however, after the hypoxic stimulus, the increase is marginal in comparison to baseline (p = 0.19) (Fig. 1a-b). αSMA gene (ACTA2) expression increases in control fibroblasts at 12, 24, 48, 72 and 96 h, with a peak after 48 h which returns to baseline level at 96 h (Fig. 1c). In IPF cells, αSMA expression is higher than in controls and the level remains high even after 96 h (Fig. 1c) (Table 2). These results indicate that hypoxia induces myofibroblast differentiation, this phenotype is still present in conditions with available oxygen in IPF.

Active hypoxia signaling in IPF derived fibroblasts by HIF-1α & HIF-2α but no HIF-3α
For decades, reports have established that hypoxic condition is associated with the production and accumulation of ECM molecules in fibroblasts of different tissues [13–18]. To evaluate whether hypoxia signaling may be a factor in ameliorating the differentiation process, HIFs were measured. The basal levels of proteins HIF-1α, HIF-2α and HIF-3α showed statistically significant differences, as it is shown in Fig. 2. HIF-1α is increased in IPF (p = 0.05) (Fig. 2 a) as well as HIF-2α (p = 0.004) (Fig. 2 b), while HIF-3α presents a different behavior with...
more HIF-3α protein in controls (p = 0.04) (Fig. 2 c). These results correlate with the differences in the expression of αSMA in basal conditions because HIF-1α and -2α participate in the activation of hypoxia signaling while HIF-3α has been reported that regulates this pathway negatively [19].

In addition, it was evaluated the dynamics of the response and it was found that when controls are exposed to hypoxia for 48 h, these cells increase their amount of HIF-1α and HIF-2α proteins with respect to normoxia (Fig. 3 a, b, d, e). However, in IPF, the amount of these two proteins showed a slightly increase (Fig. 3 a, d).

**Table 2** P-values of gene expression (hypoxia vs basal conditions)

| Gene | 12  | 24  | 48  | 72  | 96hrs |
|------|-----|-----|-----|-----|-------|
| Control | | | | | |
| HIF1 | 0.032 | 0.047 | 0.002 | <0.001 | 0.040 |
| HIF2 | 0.011 | <0.001 | <0.001 | <0.001 | <0.001 |
| ACTA | 0.001 | 0.001 | <0.007 | 0.002 | <0.001 |
| IPF  | | | | | |
| HIF1 | 0.010 | <0.001 | <0.001 | <0.001 | 0.136 |
| HIF2 | 0.037 | <0.001 | <0.001 | <0.001 | 0.004 |
| ACTA | 0.001 | <0.001 | <0.001 | <0.001 | <0.001 |

Therefore, the levels of these proteins come from a previous stimulus and remain in cells derived from IPF.

By qPCR, gene expression in control fibroblasts displays, after 12, 24, 48, 72 and 96 h of exposure to hypoxia (1% O2), a peak after 48 h for HIF-1α and at 72 for HIF-2α (EPAS1) (Fig. 3 c, f). IPF cells present a peak after 24 h in both (Fig. 3 c, f). Table 2 shows statistical differences between each different time slot and baseline (0 h). In all cases, there was a significantly increased expression of the genes in hypoxic fibroblasts when compared to basal either for controls or for IPF (p < 0.05). Table 3 indicates intergroup comparisons (normal vs IPF), which are statistically significant for HIF-1 and HIF2 (EPAS1) at 12, 24 and 72 h. In most cases, IPF cells show higher levels in response to hypoxia and remain longer. Immunocytochemistry in fibroblast supports the findings for the expression of HIF-2α (Fig. 3 g). Summarizing the results, there was an increased expression of hypoxia signaling with higher levels for IPF cell lines, mainly by HIF-2α.

**HIF-3α hypermethylation could be responsible for augment myofibroblasts differentiation in IPF**

HIF-3α is the less studied isoform, this gene produces a large number of variants by alternative splicing [19–21].
It has been accepted that the majority of these variants act as negative feedback regulators of the activity of HIF-1α and HIF-2α [14, 18–21]. According to this, the increase in the differentiation could be as a result of significant lower levels of HIF-3α protein in IPF compared to controls (p = 0.04) (Fig. 2 c). The results were even more striking in hypoxia, because in the HIF-3α promoter region there are hypoxia response elements (HRE), characteristic of the genes that are targeted by HIF-1α [14–17]. In controls, it exhibits a similar way of HIF (1 and 2) proteins, these cells increase the amount of HIF-3α but in IPF their levels remain unchanged (Fig. 4a-c). Unfortunately, for HIF3a, there is no data of PCR amplification for any sample most likely due to the probe used.

Hypoxia induces global DNA hypermethylation [18, 22]. For example, in prostate cancer, HIF-3α gene has been found hypermethylated compared to healthy tissue from the same patient and this promotes carcinogenesis [23]. Following these observations, it was tested if promoter methylation could be the reason for the decrease in HIF-3α. In the demethylation assay with 5-Aza, two IPF lines without response were tested. Aza treatment increased the levels of HIF-3α protein, suggesting that the lack of hIF3alpha is due to hypermethylation (Fig. 4d, e).

**Integrated evaluation of hypoxia-regulated transcription factors expression in tissue from IPF**

For HIF-1α expression in tissue samples, no differences were encountered in fibroblasts; however, there is a differential distribution of positive cell percentage in idiopathic fibrosis cases in comparison to control lung tissues. Though fibroblasts in tissue samples from both idiopathic fibrosis cases and normal control tissues express HIF-1α, a greater number of idiopathic fibrosis cases have medium or high percentage of positive cells (Fig. 5a, c). From the ranges of positive cells employed for the statistical analysis, the 10–50% range comprises cases with relatively low and medium percentage of positive cells. Setting different thresholds to stratify cases that separate this range at 25% percent, it emphasizes the differential distribution of HIF-1α positive fibroblasts and reflects a trend towards a greater expression of this protein among idiopathic fibrosis cases (p = 0.06) (Fig. 5a).

An evident increase in HIF-2α protein expression in fibroblasts from tissue samples was observed in idiopathic...
fibrosis in comparison to normal control tissue \( (p = 0.0011) \) (Fig. 5d, f). However, a trend towards an increase in HIF-2\(\alpha\) expression was also encountered when comparing tissue samples from other pulmonary inflammatory conditions to normal control tissue \( (p = 0.0878) \) (Fig. 5e, f). Further, for tisular fibroblasts, there was no difference in expression between idiopathic fibrosis and other inflammatory conditions \( (p = 0.335) \) (Fig. 5d, e). Significant differences in the expression of this factor were also observed in some inflammatory cells in both subsets of disease when compared to controls: higher percentage of positive plasma cells in idiopathic fibrosis tissues vs normal control \( (p = 0.0429) \), and higher percentage of positive plasma cells in other inflammatory lung conditions vs normal control \( (p = 0.0242) \).

For HIF-3\(\alpha\) the only significant differences in expression were observed in the septum wall. There was high expression in controls and loss of the stain in the remaining non-lesional lung tissue in idiopathic fibrosis cases \( (p = 0.0151) \) (Fig. 5g, h, i). This difference remained statistically significant between idiopathic fibrosis cases vs other inflammatory conditions \( (p = 0.0470) \) (Fig. 5g, h). In contrast to HIF-2\(\alpha\) expression for HIF-3\(\alpha\) there were no differences in fibroblasts or inflammatory cells, but there was a trend towards lower expression in the epithelial cells alone when comparing their HIF-3\(\alpha\) expression between controls and idiopathic fibrosis cases (hyperplastic epithelial cells within the lesion) \( (p = 0.0688) \) (Fig. 5g, i).

**Discussion**

Reduced oxygen tension is observed in many lung disorders, in the context of IPF is considered as a driving force in the progression of the disease. Above all, hypoxic conditions are getting involved in fibroblast foci formation that are the distinctive histopathological feature of this disease. The main objective of this work was to investigate the possible role of hypoxia-regulated transcription factors (HIFs) in fibroblast activation of idiopathic pulmonary fibrosis and age-related controls. On this paper, the analysis of all the factors involved by Westen blot, PCR, IHC and ICC were integrated. Previous studies have already reported that hypoxia promotes myofibroblast differentiation, in primary human AEC cells it induces the expression of \( \alpha\)SMA and decreases

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**Table 3** P-values of gene expression after hypoxia exposure (IPF vs control)

| Gene | 12   | 24   | 48   | 72   | 96 hrs |
|------|------|------|------|------|--------|
| HIF1 | 0.025| <0.001| 0.006| <0.001| 0.046  |
| HIF2 | 0.012| <0.001| 0.018| <0.001| 0.114  |
| ACTA | 0.005| <0.001| <0.001| <0.001| <0.001 |
the expression of E-cadherin [24]. In fibroblasts, this differentiation is through a MMP2-mediated pathway [18, 25, 26]. These results reinforce previous findings, HIF-1α and HIF-2α accumulation contributes to myofibroblast differentiation, as demonstrated in the increased αSMA protein expression in the control group after hypoxia. In the context of IPF, hypoxia signaling is still present in spite of having available oxygen. This indicates that IPF derived fibroblasts are probably working with anaerobic metabolism. There have been found alterations in metabolic pathways related to energetic metabolism in lungs with IPF [27]. This is also supported by other reports that describe a high production of lactate and it leads to assume that these fibroblasts probably have a similar metabolism such as those of cancer [28].

An interesting finding is the involvement of HIF-2α observed at all levels, mRNA and protein expression; in higher magnitude than that of HIF-1α. In congruency with this idea, Lin Q. et al. propose that HIF-1α plays an important role in response to acute hypoxia, while HIF-2α does so against chronic hypoxia [29]. HIF-1α and HIF-2α isoforms show differences in the subset of genes they activate, an example of this could be in the work of Hanna C. et al. in which they observed that HIF-2α plays a significant role in the expression of collagen in human mesangial cells in conditions of normoxia, so they hypothesize that HIF-2α is more important than HIF-1α in normoxic glomerular fibrogenesis [30]. Furthermore, hypoxia potentially stimulates miR-210 expression via HIF-2α, and that high miR-210 expression in turn drives fibroblasts proliferation [9]. All these data correlate with results of the study showing that the expression of HIF-2α is higher and indicate the phenotype seems to be constitutively present in IPF fibroblasts.

Regarding to the HIF-3α isoform, in this work, it was observed that HIF-3α is present in controls, whereas in IPF it is diminished. These finding match with the data of transcriptomic that show lower expression of HIF3α in IPF lungs [31]. HIF-3α is considered a primary factor in alveolarization during lung development, an example of the potent inhibitory effect it has on HIF1α and HIF-2α is that overexpressing mice show a post-pseudoglandular branching defect with a reduced number of airspaces and a clear reduction in the number of alveolar type I and type II cells [32]. This overexpression recapitulates the loss of these factors in lung maturation in newborn.

Due to the fact that there are different transcripts associated with this gene, expression levels of these variants depend on multiple conditions, they can vary depending on the stage of development, for example: HIF3α4 and HIF3α7 are expressed in adult tissues and HIF3α4 in fetal tissue [13, 33]. The expression of this gene by qPCR is not found in the results of this study, while by western blot the polyclonal antibody is able to recognize them, which it can explain this phenomenon at least in part. In lungs of patients with IPF, no HIF-3α expression was observed, maybe due to the low amount of the protein in conjunction with the abundance of collagen and sparsity of fibroblasts. It was observed a high expression of this factor in the septum wall of healthy lung
tissue by immunohistochemistry, however, little is known about this protein and much less in adult human lung.

In the same vein, the transcription factors induced by hypoxia have a central role in the distinct phenotype that manifests fibroblast derived from IPF and show an altered response to stress [34, 35]. Therefore, it is propose that αSMA gene expression, at least in part, is determined by the activity of HIF 1α and 2α, in IPF, this increase is greater perhaps due to the lack of regulation of HIF3α. In IPF, hypoxia hypermethylation could be one of the conditions by which the pathways involved in the response to stress could be altered. One of the limitations is the lack of significance difference in the IPF lines treated with the demethylating agent, because this treatment partially recovers HIF3α expression in IPF derived fibroblast. So probably some other mechanisms are involved in the reduction of HIF-3α which needs to be investigated more thoroughly.

Conclusions
Our data, altogether, reinforce the idea that hypoxia is a determining factor in the development and progression of fibrosis. We have showed a correlation between over-activation in myofibroblast differentiation as a result of the increase in HIF-1α and HIF-2α. Also, it is noted that IPF fibroblasts have a decrease in HIF-3α which could be related to its hypermethylation and this means a possible mechanism of susceptibility in patients with IPF.
Abbreviations
S-Aza: 5-Aza-2'-deoxycytidine; BCA: Bicinchoninic acid; ECM: Extracellular matrix; HIF: Hypoxia inducible factor; HRE: Hypoxia response elements; ICC: Immunocytochemistry; IHC: Immunohistochemistry; IPF: Idiopathic pulmonary fibrosis; MMP2: Matrix metalloproteinase-2; PAGE: Polyacrylamide gels; PBS: Phosphate-buffered saline; TMA: Tissue microarray; VHL: Von Hippel-Lindau; αSMA: Alpha smooth muscle actin

Acknowledgments
Laura Lorena Jiménez Sánchez as a student of the master’s program in Ciencias Químico biológicas of the Escuela Nacional de Ciencias Biológicas of the Instituto Politécnico Nacional (IPN) received the grant 619197 from Consejo Nacional de Ciencia y Tecnología, México (Conacyt).

Author’s contribution
AAG & YR conceived and designed the experiments. YR, LLJS & GGA carried out the western blots. AAG, LLJS, MCL, MLTE, VHOR & CC participated in the PCRs and performed the statistical analysis. JC, FTM & YR participated in the 5-aza experiments. HAMM, RVC, HSC & EFS carried out the immunoadsays. JZ participated in the design of the study. AAG, GGA, JZ & YR were a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Funding
No funding was received.

Availability of data and materials
Not applicable.

Ethics approval and consent to participate
Approval for this study was obtained by the institutional review board at Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas (INER, Mexico City, Mexico).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 8 January 2019 Accepted: 14 June 2019
Published online: 24 June 2019

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