

Usefulness of Bronchoscopic Probe-Based Confocal Laser Endomicroscopy in the Diagnosis of *Pneumocystis jirovecii* Pneumonia

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Key Words

Alveolscopy · Human immunodeficiency virus · Opportunistic respiratory infections

Abstract

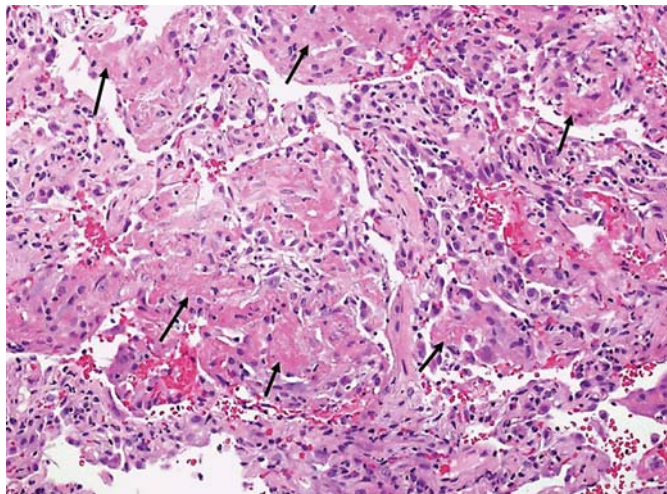
Background: Probe-based confocal laser endomicroscopy (pCLE) is a novel technique that provides in vivo microscopic imaging of the distal lung. We hypothesized that the intra-alveolar exudates characterizing *Pneumocystis jirovecii* pneumonia (PJP) can be identified by pCLE in vivo and help in its diagnosis. **Objectives:** We aimed to assess the usefulness of pCLE for the in vivo diagnosis of PJP. **Methods:** Thirty-two human immunodeficiency virus (HIV)-positive patients with new pulmonary infiltrates and fever were studied using pCLE. Real-time alveolar images were recorded during the bronchoscopy for off-line analysis by two independent observers. Bronchoalveolar lavage samples were also obtained and processed for microbiology and cytological evaluation, including Grocott stain for *P. jirovecii*. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of pCLE for the diagnosis of PJP in these patients

were calculated. **Results:** Fourteen patients (44%) were confirmed to have PJP by cultures/staining. pCLE was well tolerated in all patients. It identified intra-alveolar exudates in 13 of them (41%), where 11 of them (85%) had positive Grocott stain for *P. jirovecii*, with 93% concordance between observers. Sensitivity, specificity, PPV and NPV of pCLE for the diagnosis of PJP were 79, 89, 85 and 84%, respectively. In smokers, these figures improved to be 92, 88, 85 and 94%. **Conclusions:** pCLE is a quick and safe procedure for on-site diagnosis of PJP in HIV+ patients with excellent specificity and sensitivity mainly in smokers.

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Introduction

Pneumocystis jirovecii pneumonia (PJP) is one of the most prevalent opportunistic infections in patients infected with the human immunodeficiency virus (HIV) [1]. Patients with PJP often complain of subacute symptoms and present with interstitial infiltrates on chest X-ray and severe gas exchange impairment [2]. If not diag-



Color version available online

Fig. 1. Characteristic intra-alveolar exudate in PJP (hematoxylin-eosin) formed by proteins, dead cells and *P. jirovecii* (arrows). HE. $\times 200$.

nosed and treated promptly, PJP may progress to severe respiratory failure requiring mechanical ventilation and, eventually, death [3]. On-site PJP diagnosis is, therefore, of great clinical relevance [4].

In clinical practice, the diagnosis of PJP may be confounded by the fact that symptoms and chest X-ray findings are similar to those of bacterial pneumonia, also frequent in HIV+ patients. The definitive PJP diagnosis requires the demonstration of *P. jirovecii* in respiratory samples using specific silver staining, like Gomori or Grocott [2] or direct immunofluorescence [5]. However, these techniques are time-consuming and delay PJP diagnosis by 24–72 h, thus delaying the initiation of specific therapy (hence, worsening prognosis) or forcing the start of empiric therapy (with the risk of undesired side effects) [6]. An alternative to these diagnostic techniques, available only in some centers, is the use of molecular techniques to identify *P. jirovecii* DNA in respiratory samples by polymerase chain reaction (PCR) [7]. However, this is an expensive procedure that is also time-consuming. New and more direct diagnostic tools are therefore desirable for the early diagnosis of PJP [4].

Probe-based confocal laser endomicroscopy (pCLE) is a new minimally invasive technique that provides real-time in vivo microscopic imaging of the distal lung [3]. It involves the advancement of a miniprobe through the working channel of the fiberoptic bronchoscope. A laser beam at the end of the miniprobe elicits fluorescence of the tissue surface which is recorded in the system and analyzed off-line [8]. As we have shown recently [9],

pCLE can image and quantify the elastin network of the airways including alveolar ducts, alveolar entrance rings and extra-alveolar microvessels (alveoloscopy), as well as alveolar macrophages in vivo [3].

Considering that PJP is characterized by the presence of intra-alveolar foamy exudates formed by a conglomeration of proteins, dead cells and non-staining *P. jirovecii* (fig. 1) [10, 11], and that they are highly sensitive and specific for PJP [12], we hypothesized that pCLE might have the potential to detect these exudates and, therefore, diagnose PJP in vivo, on site. We explored this hypothesis by determining the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of pCLE in the diagnosis of PJP in HIV+ patients.

Materials and Methods

Study Design

Patients with HIV acutely admitted to our hospital (Hospital Universitari Son Espases, Palma de Mallorca, Spain) with new uni- or bilateral pulmonary infiltrates and fever, from November 2008 to August 2013, were invited to participate. Exclusion criteria were severe acute respiratory failure ($\text{PaO}_2/\text{FiO}_2 < 250$), coagulation disorder (platelets $< 50,000 \text{ cell/mm}^3$, prothrombin time test $< 50\%$) and/or general contraindications for bronchoscopy according to international guideline recommendations [13]. All participants voluntarily signed their informed consent. The study protocol had been previously approved by the local Ethics Committee.

Patient Characterization

Complete physical examination, blood cell count, general biochemistry, arterial blood gases and chest X-ray (or computed tomography with intravenous contrast if clinically indicated) were assessed in all patients. Antibiotic therapy started before bronchoscopy (if any) was also recorded.

Bronchoscopy

Fiberoptic bronchoscope was performed using a Pentax, EB-1570K endoscope (Tokyo, Japan). Bronchial aspirate (BAS) and bronchoalveolar lavage (BAL) samples were obtained following international recommendations [14]. Briefly, BAL was performed instilling 100–125 ml of normal saline in 25-ml aliquots in affected segments (mostly middle lobe and lingula) [14].

Microbiologic Determinations

Both BAS and BAL samples were processed both for microbiology (Gram and Ziehl-Nielsen stain and bacterial, fungal, viral and mycobacterium cultures) and cytological evaluation (total and differential cell count and Grocott stain for *P. jirovecii* in BAL samples) and direct immunofluorescence. PCR for PJP was not performed.

Probe-Based Confocal Laser Endomicroscopy

pCLE (Cellvizio[®]-Lung, 488 nm excitation, Mauna Kea Technologies, Paris, France) was used, as previously described by Thi-

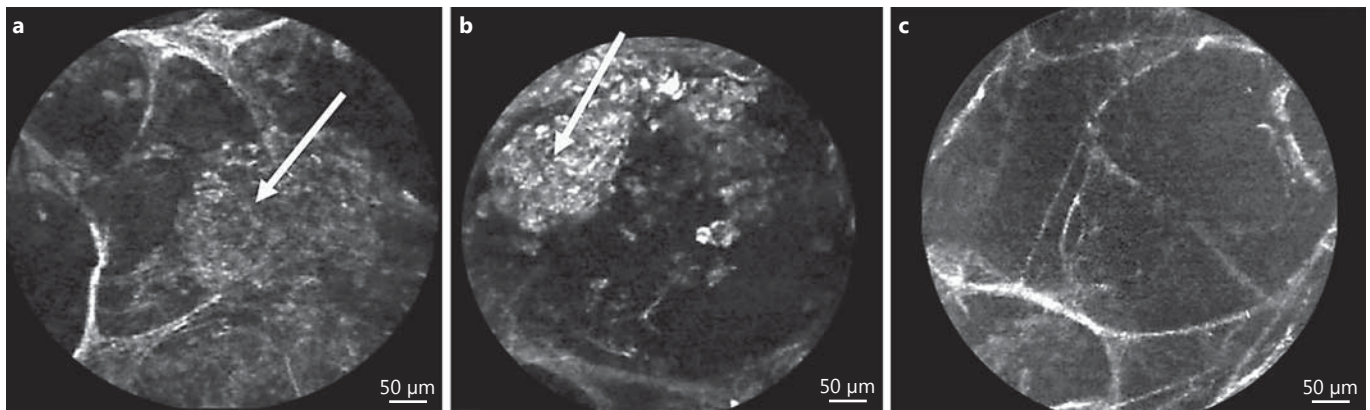


Fig. 2. Intra-alveolar exudates (arrows) detected by pCLE in 2 patients with PJP (a, b). Normal pCLE (c).

Table 1. Characteristics of the 32 patients and the BAL cellular counts

	Presence of <i>P. jirovecii</i>		p value
	yes (n = 14; 43.8%)	no (n = 18; 56.2%)	
Age, years	43.3±8.3	43±8.8	0.629
Gender			0.554
Male	11 (78.6)	15 (83.3)	
Female	3 (21.4)	3 (16.7)	
Smoking history			0.24
Smoker	12 (85.7)	17 (94.4)	
Nonsmoker	2 (14.3)	1 (5.6)	
PaO ₂	67.6±21.9	64.9±21.5	0.959
FiO ₂ %	42.6±34.5	26.3±20.4	0.872
HIV stage ^a			0.011*
Stage C	14 (100)	11 (61.1)	
Stage B	0	4 (22.2)	
Stage A	0	3 (16.7)	
Median CD4 count, cells/mm ³ (IQR)	30 (21–50)	159 (134.25–255.25)	0.0001*
Median viral count, copies/ml (IQR)	148,800 (76,483.25–408,752.75)	4,103.5 (248.5–168,147.5)	0.016*
BAL			
Lymphocytes, %	17.3±15	13.5±8.2	0.829
Macrophages, %	64±21	64.8±28.6	0.766
PMN, %	18.3±17.8	23±31	0.733

Figures are mean ± SD or numbers with percentages in parentheses. PMN = Polymorphonuclear neutrophils; FiO₂ = fraction of inspired oxygen; PaO₂ = arterial oxygen tension; IQR = interquartile range (percentile 25–75). * p < 0.05.

^a According to CDC staging.

berville et al. [3] and Cosio et al. [9]. In brief, the bronchoscope is wedged into the smallest reachable bronchi and a Confocal Mini-probe™ (AlveoFlex, 1.4 mm in diameter, Paris, France) introduced through its working channel and advanced into the distal bronchiole until the alveolar space is seen. Then, the probe is pulled back slightly to minimize compression [3]. Alveolar images were obtained in 4 different lung segments among those with most radiological abnormalities. Real-time alveolar images were continuously recorded during the procedure and stored for off-

line analysis, as detailed below. After bronchoscopy, a plain chest X-ray was obtained routinely. The duration of pCLE procedure was recorded.

Off-Line pCLE Analysis

We considered that the patient had intra-alveolar exudates if at least one homogeneous intra-alveolar infiltrate was observed off-line (fig. 2) by two independent observers. In case of discordance, a final consensus decision was reached after a new analysis of the

Table 2. Individual microbiologic and pCLE results

Patient ID	Microbiologic cultures	Presence of intra-alveolar exudates (pCLE)		
		two observers	1st observer	2nd observer
1	BAS: <i>E. coli</i>	-	-	-
2	BAS: <i>S. aureus</i> sensitive to methicillin + <i>Candida parasilopsis</i>	-	-	-
3	BAS and BAL: <i>P. jirovecci</i>	+	+	+
4	BAS and BAL: <i>S. pneumoniae</i>	-	-	-
5	Negative culture	-	-	-
6	BAS and BAL: <i>M. tuberculosis</i> and <i>Candida albicans</i>	-	-	-
7	BAS and BAL: <i>P. jirovecci</i>	+	+	+
8	BAS and BAL: CMV	-	-	-
9	BAS and BAL: <i>M. tuberculosis</i>	+	+	+
10	BAS and BAL: <i>M. tuberculosis</i>	-	-	-
11	BAS and BAL: <i>P. jirovecci</i>	+	+	+
12	Transbronchial biopsy: <i>Mycobacterium avium-intracellulare</i>	-	-	-
13	Negative culture	-	-	-
14	Negative culture	-	-	-
15	Negative culture	-	-	-
16	BAS and BAL: <i>K. pneumoniae</i>	-	-	-
17	BAS and BAL: <i>P. jirovecci</i>	-	-	-
18	Negative culture	+	+	+
19	BAS and BAL: <i>P. jirovecci</i>	+	+	+
20	BAS and BAL: <i>P. jirovecci</i>	+	+	+
21	BAS and BAL: <i>H. influenzae</i>	-	-	-
22	BAS and BAL: <i>P. jirovecci</i>	+	+	+
23	BAS and BAL: <i>P. jirovecci</i>	+	+	+
24	BAS and BAL: <i>P. jirovecci</i>	+	+	+
25	BAS and BAL: <i>P. jirovecci</i>	-	-	+
26	Negative culture	-	-	-
27	Negative culture	-	-	-
28	BAS and BAL: <i>P. jirovecci</i>	-	-	-
29	Negative culture	-	-	-
30	BAS and BAL: <i>P. jirovecci</i>	+	+	+
31	BAS and BAL: <i>P. jirovecci</i>	+	+	+
32	BAS and BAL: <i>P. jirovecci</i>	+	+	+

CMV = Cytomegalovirus.

imaging sequence by the two observers together. Build-in software (MedViewer® 1.1.1; Mauna Kea Technologies, Paris, France) was used for the measurement of the extra-alveolar vessel diameter, alveolar diameter, alveolar elastic fiber thickness and alveolar fluorescence intensity as previously described [3, 8, 9].

Statistical Analysis

Results are presented as mean ± standard deviation (SD) or number (percentage) as appropriate. Characteristics of patients with and without PJP were compared using the Mann-Whitney test or χ^2 test as appropriate, unless otherwise stated. Concordance between the two pCLE observers was assessed using the kappa index. Receiver operating characteristic (ROC) curve was constructed and the area under the curve (AUC) determined. Sensitivity,

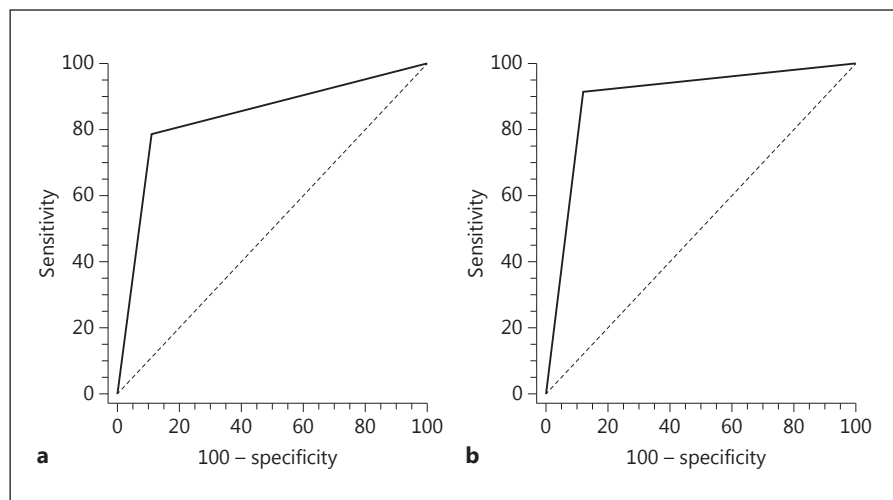
specificity, PPV and NPV of pCLE for the diagnosis of PJP in these patients were also calculated. A p value <0.05 (two-tailed) was considered significant. Analysis was performed using MedCalc® (version 9.2.1.0, Acacialaan 22, Ostend, Belgium).

Results

Clinical Characteristics of Participants

We studied 32 HIV+ patients (26 males and 6 females) hospitalized because of acute febrile pulmonary infiltrates. Table 1 presents their main clinical characteristics.

Fig. 3. a ROC of pCLE to detect intra-alveolar exudates in all HIV+ patients (AUC = 0.837, $p = 0.0001$). **b** ROC of pCLE to detect intra-alveolar exudates in HIV+ patients being smokers (AUC = 0.900, $p = 0.0001$).



Mean age was 43 years and there was a preponderance of males. Most patients were current smokers. Pulmonary gas exchange was moderately to severely impaired (table 1). As expected, we found a higher HIV stage, lower CD4 lymphocytes and higher HIV copies in patients with PJP ($n = 14$) than in those without ($n = 18$) (table 1).

Table 2 shows the microbiological results of BAS and BAL in each individual. Grocott stain was positive for *P. jirovecii* in 14 patients (44%), cultures/staining were negative in 8 patients (25%) and other micro-organisms were detected in the remaining 10 patients (31%). We found 2 false positives (11%) in the 18 patients (56%) without PJP.

Bronchoscopy and pCLE

Bronchoscopy and pCLE were well tolerated by all participants, and no patient developed any significant complication during or after the procedures. The mean duration of pCLE was 3.9 ± 2.7 min (max. 13 min in 1 case). Table 2 also presents pCLE presence/absence of alveolar exudates in each individual. Concordance between the two observers was 93%. pCLE identified intra-alveolar exudates in 13 patients (41%) of which 11 (85%) had positive Grocott stain for *P. jirovecii*. In 3 patients out of the 14 (21%) with positive *P. jirovecii* stains (ID 17, 25 and 28 in table 2), pCLE did not detect intra-alveolar exudates. Two of these 3 patients were nonsmokers, and all of them had started treatment for *P. jirovecii* [sulfamethoxazole/trimethoprim 800/160 mg plus corticosteroids (methylprednisolone 40 mg/24 h)] ≥ 48 h before pCLE. The sensitivity, specificity, PPV and NPV of pCLE for the identification of intra-alveolar exudates in PJP were 79, 89, 85 and 84%, respectively (AUC = 0.837, $p =$

Table 3. The frequency of both positive and negative pCLE and *P. jirovecii* detected microbiologically among the smokers

	pCLE		Total
	negative	positive	
Presence of <i>P. jirovecii</i>			
No	15 (93.7%)	2 (15%)	17 (58.6%)
Yes	1 (6.3%)	11 (85%)	12 (41.4%)
Total	16 (55.2%)	13 (44.8%)	29 (100%)
Fisher's exact test (p value)			<0.0001*

0.0001; fig. 3a). In smokers only ($n = 29$, 91%), these figures were 92, 88, 85 and 94%, respectively (AUC = 0.9, $p = 0.0001$; fig. 3b). Table 3 shows the frequencies of positive and negative pCLE detection of intra-alveolar exudates among the smokers group.

Tables 4 and 5 show several morphometric measurements obtained using pCLE regarding smoking history or pneumocystis infection, respectively. There were no significant differences except for the alveolar diameter that was lower in patients with PJP (table 5).

Discussion

The results of this study indicate that pCLE is a safe and efficacious procedure for real-time, on-site, in vivo diagnosis of PJP in HIV+ patients.

Table 4. Morphometric measurements

Morphometric measurements	Smokers (n = 29)	Nonsmokers (n = 3)	p value
Alveolar diameter, μm	257.97 \pm 33.3	239 \pm 65.8	0.539
Alveolar elastic fiber diameter, μm	13.4 \pm 2.4	12.4 \pm 0.9	0.348
Extra-alveolar vessel diameter, μm	131.04 \pm 43.1	135.03 \pm 49.3	0.841
Alveolar intensity, μm	216.8 \pm 115.2	145.6 \pm 121.4	0.349
Macrophages, n (%)	27 (84.4)	2 (6.2)	0.263

Table 5. Morphometric measurements among the studied cohort of patients

Morphometric measurements	Presence of <i>P. jirovecii</i>		p value
	yes (n = 14)	no (n = 18)	
Alveolar diameter, μm	236.6 \pm 39.8	271.5 \pm 24.9	0.028*
Alveolar elastic fiber diameter, μm	12.4 \pm 1.9	14 \pm 2.4	0.074
Extra-alveolar vessel diameter, μm	131.7 \pm 54.7	131.2 \pm 33.7	0.575
Alveolar intensity	188.3 \pm 142.4	227.1 \pm 90.6	0.224
Macrophages, n (%)	12 (37.5)	17 (53.13)	0.408

* p < 0.05.

Previous Studies

Previous studies used pCLE to investigate interstitial lung disease, alveolar proteinosis, drug-induced (amiodarone) pneumonitis, lung cancer [15–18] and, very recently, chronic obstructive pulmonary disease [9]. To the best of our knowledge, this is the first study to investigate the potential clinical usefulness of pCLE in the diagnosis of PJP in HIV+ patients.

Safety of the Procedure

Previous studies have reported a minor risk of pneumothorax and chest pain [15] or mild bleeding [19] following pCLE. This was not the case in any of the patients included in our study. Of note, however, we excluded patients with severe acute respiratory failure and mechanical ventilation. Therefore, our results cannot be extended directly to more severely ill HIV+ patients.

Usefulness of pCLE for the Diagnosis of PJP

Despite the availability of highly active antiretroviral therapy and the widespread use of PJP prophylaxis [20], the latter still is one of the commonest opportunistic infections in HIV+ patients [21, 22]. Additionally, the high mortality of patients with PJP requiring mechanical ventilation remains unchanged (50–60%) [23]. To improve survival, early empirical treatment with trimethoprim/sulfamethoxazole is generally recommended [24]. Yet, this can have severe adverse effects which are considered

high in HIV+ patients ranging between 20 and 85% [6, 25]. Importantly, there is no currently available rapid test for the on-site accurate diagnosis of PJP. The classical diagnostic method (Gomori, Grocott silver staining) usually requires 48 h. Immunofluorescence and molecular tests such as PCR are not available in all centers and final results also need several hours or days.

Our study shows that pCLE allows the identification of alveolar exudates that closely resemble those found in lung biopsies (fig. 2) and that its presence is highly specific for PJP (89%), with a sensitivity of 79%, a PPV of 85% and an NPV of 84%. Several factors can contribute to explain the absence of pCLE exudates in 3 patients with PJP (false negatives). First, they had started empiric antibiotic treatment 3–5 days before pCLE; this can improve the clinical condition of mild to moderate PJP cases [23] and decrease the diagnostic yield of other methods, such as PCR [7, 26]. Second, these 3 false-negative patients also received treatment with corticosteroids, known to accelerate the resolution of PJP, particularly if initiated during the first 72 h of hospitalization [27–29]. Third, interestingly, 2 of these 3 patients were nonsmokers. Whether or not smoking facilitates the formation of alveolar exudates in PJP patients is unknown. Yet, it is well established that the number of alveolar macrophages (an integral component of these exudates) is higher in smokers than nonsmokers [30], and that smoking increases their autofluorescence [8] and surfactant protein A both in plasma and

sputum [31]. The latter is potentially relevant in the context of this study since Salaün et al. [16] and Koziel et al. [32] reported that *P. jirovecii* infection was also associated with increased levels of surfactant protein A in the alveoli, which is part of the proteinaceous material of intra-alveolar exudates [11]. Thus, the combination of these factors can contribute to explain a better diagnostic performance of pCLE in smokers (sensitivity 92%, specificity 88%, PPV 85% and NPV 94%).

We found no morphometric differences between smokers and nonsmokers (table 3) and lower alveolar diameter in patients with PJP (table 4). The latter could be due to some degree of fibrosis that has been reported in lung biopsies in these patients [33].

Clinical Implications

All in all, our observations indicate that pCLE offers the possibility of a rapid on-site diagnosis and consequently that treatment can be initiated quickly based on a specific diagnosis that may improve prognosis. Likewise, we show that pCLE can also contribute to exclude this diagnosis, thus forcing the clinician to consider the presence of other microbiologic agents, such as bacterial or mycobacterial infection that require a different therapeutic strategy. There are other lesser invasive methods than bronchoscopy that has a good yield of 90% such as oral washings with PCR-based diagnostics or detection of blood β -glucan [34, 35]. However they are not fully available in all hospitals and β -glucan cannot rule out other microorganisms, especially fungal infection [36] as well as a risk of detecting subclinical colonization by *P. jirovecii* using PCR [37]. Bronchoscopy is a minimally invasive procedure that allows pCLE but can also obtain respiratory secretions and bronchoalveolar lavage allowing their analyses identifying other potential microorganisms. We do not defend the replacement of lesser invasive techniques but we stress a new inclusion of pCLE indication besides pulmonary aspergillosis, alveolar proteinosis, drug pneumonitis and lung cancer [15–18, 38, 39].

Strengths and Limitations

The present study is the first to report the usefulness of pCLE in the diagnosis of PJP but has some limitations that deserve comment. First, practically, all parts of the lung cannot be imaged by confocal endomicroscopy; thus, alveolar exudates and PJP can be missed in some cases. However, according to the study protocol, we imaged different lung segments with most radiological abnormalities. Second, the present study includes a relatively small number of patients. This is likely to be a fortunate conse-

quence of the new antiretroviral treatments that reduces drastically the incidence of opportunistic infections in HIV patients [40]. Yet, further studies in larger cohorts are required to firmly establish the diagnostic usefulness of pCLE in this context. Third, as discussed above, our results cannot be directly extended to more severe patients under mechanical ventilation. Lastly, the present study did not include HIV– patients infected with PJP. However, PJP is more common in HIV+ rather than non-HIV immunocompromised patients owing to various factors including lack of chemoprophylaxis in these patients as well as a high percentage of patients were newly diagnosed with HIV at the time of PJP infection. Further, *P. jirovecii* prophylaxis in non-HIV immunocompromised patients appears to be more effective than in HIV infection that is associated with a decreased absolute number of PJP cases with an estimated risk reduction of 91% as being reported by Green et al. [41] and Rodriguez et al. [42].

Conclusions

This study shows that pCLE is a quick and safe procedure for the on-site diagnosis of PJP in HIV+ patients by providing in vivo ‘histological’ images of the characteristic intra-alveolar exudates, with excellent specificity and sensitivity, particularly in active smokers.

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