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10 years of prophylaxis with nebulized liposomal amphotericin B and the changing epidemiology of *Aspergillus* spp. infection in lung transplantation

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Summary

The aim of this study was to assess the outcome and tolerability of prophylactic nebulized liposomal amphotericin B (n-LAB) in lung transplant recipients (LTR) and the changing epidemiology of *Aspergillus* spp. infection and colonization**.** We performed an observational study including consecutive LTR recipients (2003–2013) undergoing n-LAB prophylaxis lifetime. A total of 412 patients were included (mean postoperative follow-up 2.56 years; IQR 1.01–4.65). Fifty-three (12.8%) patients developed 59 *Aspergillus* spp. infections, and 22 invasive aspergillosis (overall incidence 5.3%). Since 2009, person-time incidence rates of *Aspergillus* spp. colonization and infection decreased (2003–2008, 0.19; 2009–2014, 0.09; $P = 0.0007$), but species with reduced susceptibility or resistance to amphotericin significantly increased (2003–2008, 38.1% vs 2009–2014, 58.1%; $P = 0.039$). Chronic lung allograft dysfunction (CLAD) was associated with *Aspergillus* spp. colonization and infection (HR 24.4, 95% CI 14.28–41.97; $P = 0.00$). Only 2.9% of patients presented adverse effects, and 1.7% required discontinuation. Long-term administration of prophylaxis with n-LAB has proved to be tolerable and can be used for preventing *Aspergillus* spp. infection in LTR. Over the last years, the incidence of *Aspergillus* spp. colonization and infection has decreased, but species with reduced amphotericin susceptibility or resistance are emerging. CLAD is associated with *Aspergillus* spp. colonization and infection.

Introduction

Aspergillus spp. is the most common cause of invasive fungal infection in lung transplant recipients (LTR) [1]. Despite the advances in antifungal drugs, infection by *Aspergillus* spp. is associated with persistently high mortality in this population [2]. Therefore, preventive measures are preferred over treatment, but the optimal antifungal drug, use of universal or targeted prophylaxis, and duration of the prophylactic strategy remain to be determined [3].

Among available antifungal agents, amphotericin B by nebulized administration (n-AB) reaches the most distal areas of the bronchial tree while avoiding drug interactions and systemic side effects [4]. However, information is

lacking on the efficacy of n-AB, the incidence of breakthrough aspergillosis, and the impact of n-AB exposure on *Aspergillus* speciation or susceptibility to this drug [5].

Several types of n-AB preparations are available. In April 1993, we began using prophylactic nebulized B deoxycholate (n-ABD) for *Aspergillus* spp. infection in all LTR. In July 2003, n-ABD was switched to nebulized liposomal amphotericin (n-LAB) because n-ABD supply was lacking on the Spanish market. We then attempted to work out a lifetime n-LAB prophylactic strategy. The initial results were reported in a previous study [6], which included the first 104 LTR receiving prophylactic n-LAB in two centers and followed up for 12 months. The n-LAB strategy proved effective, with a 1.2% incidence of invasive aspergillosis (IA). We also observed [7] that drug concentrations after n-LAB remained high and adequate for *Aspergillus* spp. prophylaxis during 14 days, a convenient administration interval. Other publications have reported good tolerance to n-LAB [6] and an optimal safety profile [8], with no evidence of significant systemic absorption [7,9], effects on respiratory function [7], or changes in lipid content of pulmonary surfactant[10], permitting long-term administration.

After 10 years of experience with this therapy in our center, we have now set out to reassess the outcome and tolerability of prophylactic nebulized liposomal amphotericin B (n-LAB) in a large number of LTR over lengthy follow-up. In addition, we have investigated the long-term impact of prophylactic n-LAB use on the evolution of *Aspergillus* spp. infection and colonization in LTR, and associations between these infections and chronic lung allograft dysfunction (CLAD).

Materials and methods

Study setting and patient population

A retrospective, observational study was performed on all consecutive adult patients undergoing lung transplantation in Hospital Univeristari Vall d'Hebron (Barcelona, Spain) from July 2003 to July 2013, receiving lifetime n-LAB prophylaxis.

We included all patients older than 18 years who had survived more than 24 h after transplantation. All patients had been followed up for at least 1 year or until death. Cases of *Aspergillus* spp. infection were identified through the General Hospital, Microbiology, and Histopathology databases, using a standardized protocol. The study protocol was approved by the Vall d'Hebron Ethics Committee for Clinical Research.

Prophylaxis for *Aspergillus* spp. infection

Since July 2003, all patients undergoing lung transplantation in our center receive 25 mg (6 ml) of n-LAB thrice weekly for the first 60 days, 25 mg once weekly between 60 and 180 days, and 25 mg once every 2 weeks thereafter, for life. Routinely, all patients with episodes of *Aspergillus* spp. colonization and all high-risk patients (suture abnormalities, post-transplantation culture isolation of *Aspergillus* spp., CMV disease, or increased immunosuppression) are treated by maintaining or increasing the n-LAB dose to thrice weekly.

In our center, prophylaxis with azoles or echinocandins is not routinely performed, except in patients with pretransplant colonization with AB-resistant fungi (*A. terreus*, *Scedosporium* spp.) or with severe intolerance to inhaled n-LAB.

Disease definitions

Classification of *Aspergillus* spp. colonization and infection is described in Table 1. Pretransplant *Aspergillus* spp. colonization included colonization from minimum 1 year before the transplant onwards and positive intra-operative respiratory samples from the explanted organ. Ulcerative tracheobronchitis and invasive pulmonary aspergillosis (IPA) were regarded as invasive aspergillosis (IA), whereas simple tracheobronchitis, bronchial stent infection, and native-lung aspergilloma were classified as noninvasive aspergillosis (NIA). The EORTC/MSG [11] and International Society of Heart and Lung Transplantation (ISHLT) [12] criteria were used to define IA cases, and only proven and probable cases were included.

We examined factors that were associated with *Aspergillus* spp. infection in previous studies: single lung transplant, chronic gram-negative bacteria colonization, bronchial stenosis, acute rejection, CMV disease, bronchial stent, pretransplant and post-transplant *Aspergillus* spp. colonization, massive inhalation, excessive immunosuppression, abandonment of prophylaxis, and induction immunosuppression therapy [13,14]. Excessive immunosuppression was defined as an amount of corticosteroid (the equivalent of 1 mg/kg daily doses of prednisone during the last month) given as therapy or pulses, and/or treatment with thymoglobulin, basiliximab, OKT3, and total lymphoid irradiation (TLI).

Aspergillus spp. infection and colonization were categorized into early-onset, occurring <90 days after transplantation and late-onset, occurring >90 days after. [15] Therapy response was categorized as success or failure, as has been described elsewhere. [16] Mortality was considered IA-related if IA was the cause or played a major role in the patient's death, and IA-unrelated if IA played a minor or no role.

Statistics

A descriptive analysis was performed. Continuous variables are expressed as the median and range. All proportions were calculated as percentages of patients with available data. Categorical variables were analyzed using the

Table 1. Classification of *Aspergillus* spp. colonization and infection modified [6,11,12].

Colonization	Single positive bronchoalveolar lavage or bronchial aspirate; or positive bronchoalveolar lavage galactomannan test; or at least two positive sputum cultures or tracheal aspirate for *Aspergillus* spp. in asymptomatic patients with normal-appearing respiratory mucosa or absence of endobronchial lesions
Aspergillus infection	
Simple tracheobronchitis	Detection of *Aspergillus* spp. and clinical symptoms (e.g, purulent sputum production) plus bronchoscopy findings of mucus and edematous red mucosa, with bacterial infection ruled out.
Bronchial stent infection	Detection of *Aspergillus* spp. and clinical symptoms (e.g, purulent sputum production) plus bronchoscopy findings of mucus and edematous red mucosa, with bacterial infection ruled out in patients with bronchial stent
Native lung aspergilloma	An approximately spherical shadow with surrounding air, also called a fungal ball, in a pulmonary cavity, with serological or microbiological evidence that *Aspergillus* spp. is present in the material
Ulcerative/pseudomembranous tracheobronchitis	Detection of *Aspergillus* spp. with bronchial biopsy and/or bronchoscopy findings of necrotic ulcers or pseudomembrane in the anastomosis or in the tracheobronchial tree that disappeared after treatment
Invasive pulmonary aspergillosis	Detection of *Aspergillus* spp. with evidence of tissue damage on lung histopathology or radiological signs of invasive aspergillosis

chi-square test. *Aspergillus* spp. colonization and/or infection incidence rates were calculated as number of cases at risk during follow-up time. We assessed the influence of *Aspergillus* spp. colonization/infection on the time to subsequent development of CLAD and the reverse relationship with Cox proportional hazards regression: onset of CLAD and *Aspergillus* spp. colonization/infection were time-dependent covariates. Differences were considered significant at a value of $P < 0.05$.

Results

Patients and baseline characteristics

A total of 412 patients were included, and mean postoperative follow-up was 2.56 years (IQR 1.01–4.65). Clinical characteristics of the study population are reported in Table 2.

Table 2. Demographic data and patient characteristics.

Variable	Number of patients (%)
Patients	412
Age, mean (SD), years	49.9 (±11.4)
Sex, *n* (%)	
Male	257 (62.4)
Female	155 (37.6)
Pretransplant diagnosis, *n* (%)	
Idiopathic pulmonary fibrosis	159 (38.6)
Chronic obstructive pulmonary disease	152 (36.9)
Cystic fibrosis	26 (6.3)
Primary pulmonary hypertension	18 (4.4)
Bronchiectasis	17 (4.1)
Lymphangioleiomyomatosis	15 (3.6)
Others	25 (6.1)
Pretransplant *Aspergillus* colonization, *n* (%)	74 (18)
Transplant type, *n* (%)	
Double	264 (64.1)
Single	148 (35.9)

Aspergillus spp. infections

Overall, 53 (12.9%) patients developed 59 *Aspergillus* spp. infections: most patients manifested only noninvasive forms (31/412, 7.5%) and the remaining patients, invasive disease (22/412, 5.3%), which yielded a 3.6% 1-year cumulative incidence of IA. Of the 22 IA patients, 15 (3.6%) had IPA and 7 (1.7%) ulcerative tracheobronchitis. Of the 31 NIA patients, 23 (5.6%) had simple tracheobronchitis, 6 (1.5%) bronchial stent infections, and 2 (0.7%) native-lung aspergillomas (Fig. S1). Six patients (1.5%) presented 2 episodes of infection. None of the patients had disseminated infection and two patients had only extrapulmonary involvement (1 sternal osteomyelitis, 1 wound infection).

Median time from transplantation to the first *Aspergillus* spp. infection was 266 days (107–884 IQR). Based on the time, infection was diagnosed after transplantation; 50 (84.7%) were classified as late-onset (Fig. 1). Overall about half of *Aspergillus* spp. infections (30/59; 50.8%) occurred < 270 days (9 months) after transplant. Fifteen of 22 IA (68.1%) occurred <12 months after transplant (Fig. 1).

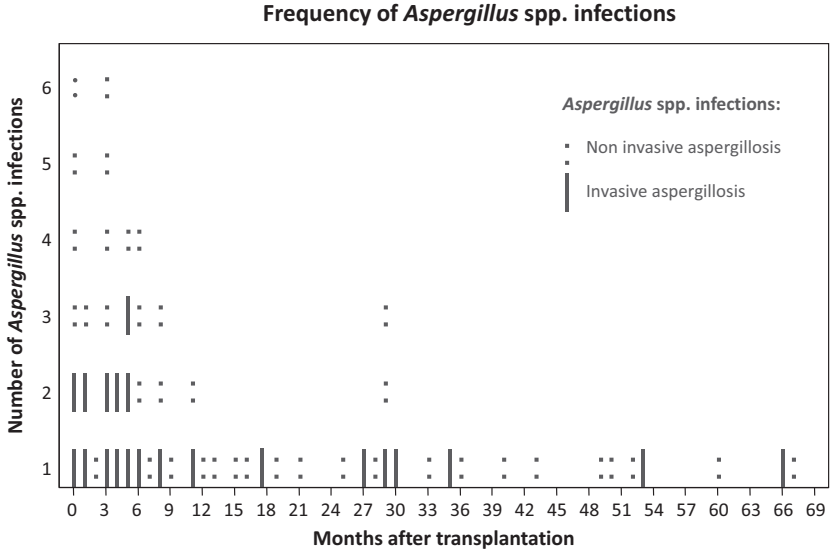


Figure 1 Onset of *Aspergillus* spp. infection after transplantation.

Findings on bronchoscopy, performed in 58 of 59 patients, are reported in Tables 3 and 4. The clinical characteristics, microbiological, bronchoscopy and radiologic findings, treatment, and outcome of IA patients are reported in Table 3. Thirteen IA patients (59.1%) met criteria for proven IA and 9 (40.9%) for probable IA.

In addition, 68 post-transplant *Aspergillus* spp. colonizations were noted in 61 LTR (14.8%). Six LTR (9.8%) developed *Aspergillus* spp. infection at any time after post-transplant colonization (only 1 IA, 1.6%). Person-time incidence rates of *Aspergillus* spp. colonization and infection were lower in the last 5 years of the study (2003–2008, 0.19; 2009–2014, 0.09; $P = 0.0007$; Fig. 2).

Tolerability

Over the 10-year study period, only 12 (2.9%) of 412 patients with lifetime prophylaxis and prolonged follow-up (2.56 years, IQR 1.01–4.65) experienced mild adverse effects associated with n-LAB. Mild, transitory breathing difficulty occurred in 8 patients (1.9%), nausea in 3 (0.7%), and dizziness in 1 (0.2%). Prophylaxis had to be stopped in 7 (1.7%) because of secondary effects, and 7 (1.7%) other patients abandoned n-LAB prophylaxis spontaneously.

Etiology

Infections due to nonfumigatus *Aspergillus* species (*A. flavus, A. niger, A. nidulans,* and *A. terreus*) were more common (35/59, 59.3%) than those caused by *A. fumigatus* (11/59, 18.6%). Mixed infections by 2 or 3 *Aspergillus* species occurred in 22% of episodes. Of interest, 1 cryptic *Aspergillus flavus* complex (*A. alliaceus*) was isolated in 1 patient with IA.

Although *Aspergillus* colonization and infection rates decreased over the last 5 years (Fig. 2), cases involving *Aspergillus* species with reduced susceptibility or resistance to amphotericin (*A. flavus*, *A. terreus,* and *A. alliaceus*) significantly increased (2003–2008, 38.1% vs 2009–2014, 58.8%; $P = 0.04$; Fig. 2).

Factors predisposing to *Aspergillus* spp. infection

Factors predisposing to *Aspergillus* spp. infection are listed in Table 5. Of note, more than half the *Aspergillus* spp. infection episodes were associated with chronic gram-negative colonization (57%), mainly *P. aeruginosa*.

No significant differences were observed between early and late IA episodes, although there was a trend toward greater frequency of association with bronchial stenosis (55.1% vs 10%, $P = 0.13$) and CLAD (42.9% vs 0%, $P = 0.01$) in late episodes. The rate of *Aspergillus* spp. infections in double LTR was similar to that of single LTR (13.6% vs 11.5%, $P = 0.6$).

Aspergillus spp. and chronic lung allograft dysfunction

Time-dependent Cox regression analysis showed that CLAD was associated with the development of *Aspergillus* spp. colonization and infection in all patients (HR 24.4, 95% CI 14.28–41.97; $P = 0.00$; Fig. 3a).

We found no time-dependent relationship between colonization and infection by *Aspergillus* spp. in general and development of CLAD (HR 0.77, 95% CI 0.44–1.34, $P = 0.3$; Fig. 3b).

Table 3. Clinical characteristics, microbiological, bronchoscopic, and radiologic findings, treatment, and outcome of patients with invasive aspergillosis (IA).

	Sex, Age, y	Transplant	Time elapsed, days	Risk factors	Type of infection	Type of IA	*Aspergillus* spp.	Bronchoscopy	Chest CT	Treatment	Death
1	M, 63	Single lung	162	8,10	IPA	Probable	*A. flavus* + *A. niger*	mucus or mucus plaques	nodules	voriconazole + anidulafungin + n-LAB	Unrelated
2	F, 64	Single lung	98	1,10	IPA	Probable	*A. alliaceus*	plaque	nodules	voriconazole + anidulafungin + n-LAB	No
3	M, 34	Double lung	2000	6,7,8,10	IPA	Proven	*A. flavus*	plaque	nodules	voriconazole + n-LAB	Related
4	M, 43	Double lung	521	1,4,8,11	IPA	Proven	*A. flavus* + *A.terreus*	erythema	cavitation	voriconazole + n-LAB	Related
5	M, 52	Double lung	831	4,11	IPA	Probable	*A. fumigatus* + *A. terreus*	erythema	nodules	voriconazole + n-LAB	No
6	M, 29	Double lung	1611	8,1	IPA	Proven	*A.fumigatus* + *A. flavus*	ulcerations	nodules	voriconazole + caspofungin + n-LAB	Related
7	M, 57	Double lung	900	8,1	IPA	Proven	*A. fumigatus*	no performed	cavitation	voriconazole + caspofungin + n-LAB	Related
8	F, 18	Double lung	883	6,7,8,10	IPA	Probable	*A. fumigatus*	mucus or mucus plaques	cavitation	voriconazole + AMB + n-LAB	Related
9	M, 55	Double lung	31	1,10	IPA	Proven	*A. fumigatus*	mucus or mucus plaques	cavitation	voriconazole + micafungin + n-LAB	Related
10	M, 57	Single lung	252	4,6,10	IPA	Proven	*A. flavus*	mucus or mucus plaques	cavitation	voriconazole + n-LAB	Related
11	M, 38	Double lung	285	8,10	IPA	Proven	*A. flavus* + *A.terreus*	plaque	cavitation	voriconazole + n-LAB	No
12	M, 60	Double lung	1050	5,8,9	IPA	Proven	*A. flavus*	plaque	new or progressive and persistent inflitrate	voriconazole + n-LAB	Related
13	M, 44	Single lung	20	1,5	IPA	Proven	*A. fumigatus* + *A. nidulans*	plaque	consolidation	AB + n-LAB	Unrelated
14	F, 33	Double lung	161	8	IPA	Probable	*A. flavus*	normal	nodules	voriconazole + n-LAB	No

Table 3. continued

	Sex, Age, y	Transplant	Time elapsed, days	Risk factors	Type of infection	Type of IA	*Aspergillus* spp.	Bronchoscopy	Chest CT	Treatment	Death
15	F, 58	Double lung	177	1,9	IPA	Probable	*A. fumigatus*	normal	nodules	voriconazole + caspofungin + n-LAB	No
16	M, 59	Double lung	203	1,2,6,10	Ulcerative TB	Proven	*A. flavus*	plaque	negative	voriconazole + n-LAB	No
17	M, 49	Double lung	17	3	Ulcerative TB	Proven	*A. flavus*	pseudomembrane formation	consolidation	voriconazole + n-LAB	Related
18	M, 51	Double lung	330	4,6,9	Ulcerative TB	Proven	*A. terreus* + *A. niger*	plaque	new or progressive and persistent inflitrate	AB + n-LAB	Unrelated
19	M, 43	Double lung	107	1,4,9	Ulcerative TB	Probable	*A. fumigatus* + *A. flavus*	plaque	new or progressive and persistent inflitrate	AB + n-LAB	Unrelated
20	M, 57	Double lung	36	3,10	Ulcerative TB	Probable	*A. terreus*	plaque	consolidation	voriconazole + n-LAB	No
21	F, 55	Double lung	121	5,10	Ulcerative TB	Probable	*A. fumigatus*	plaque	negative	voriconazole + n-LAB	Unrelated
22	F, 49	Single lung	137	5,9,10	Ulcerative TB	Probada	*A. fumigatus* + *A. terreus* + *A. niger*	plaque	negative	voriconazole + n-LAB	No

AB, ambisome (intravenous); CT, computed tomography; F, female; M, male; IA, invasive aspergillosis; IPA, invasive pulmonary aspergillosis; n-LAB, nebulized liposomial amphotericin; TB, tracheobronchitis.

Risk factors. 1: acute rejection, 2: induction immunosuppression, 3: overimmunosuppression, 4: CMV, 5: pretransplant *Aspergillus* spp. Colonization, 6: bronchial stenosis, 7: bronchial stent, 8: CLAD, 9: massive inhalation, 10: chronic gram negative bacteria colonization, 11: abandonment of prophylaxis.

Table 4. Bronchoscopy findings in invasive aspergillosis (IA) and noninvasive aspergillosis (NIA).

	Ulcerations or pseudomembrane or plaques	Mucus	Erythema	Normal	Total
IA	13 (59%)	4 (18.2%)	2 (9.1%)	2 (9.1%)	21/22
NIA	0	32 (86.4%)	3 (8.1%)	2 (5.4%)	37/37

Treatment and mortality

IA patients were treated with various antifungals until successful therapy response was achieved. Then, antifungal therapy was interrupted and n-LAB prophylaxis was restarted lifetime. (Table 3), and 60% had a successful outcome; median time to cure was 178 days (IQR 32–872). Successful therapy response was similar IA caused by potentially amphotericin-resistant species and IA by other *Aspergillus* species (57.1% vs 50.0%, respectively; $P = 0.5$). All cases of simple tracheobronchitis had successful outcomes. Patients with a bronchial stent had a poorer prognosis, showing a high rate of therapy failures (7/9, 77.8%), persistent chronic infection, and development of simple tracheobronchitis (2/9, 22.2%) or IA (1/9, 11.1%), despite treatment.

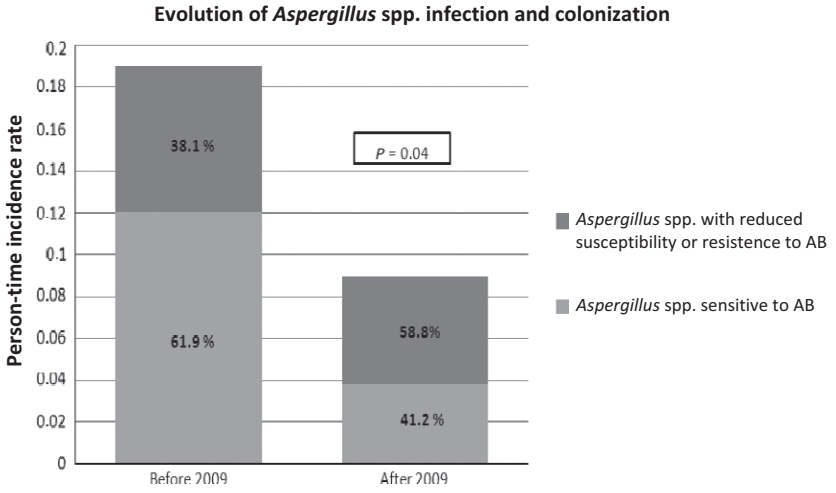
In total, 14 (63.6%) patients died after IA was detected, with 9 related (40.9%) and 5 unrelated (22.7%) deaths. Within IA forms, 8 of 15 (53.3%) patients with IPA and 1 of 7 (14.3%) with ulcerative TB presented a related death. Related mortality was 40.9% in invasive disease versus 3.2% in noninvasive forms (1 aspergilloma), and mortality was similar in IA caused by potentially amphotericin-resistant species and IA by other *Aspergillus* species (40.0% vs 42.9%, respectively; $P = 1.0$). Overall, 9 LTR (2.2%) died of *Aspergillus* spp. infection, accounting for 4.7% of the 188 deaths in these patients.

Discussion

The strengths of the present study reside in the large lung transplant population analyzed and the lengthy follow-up (compared to previous literature in this line) to reassess the outcome and tolerability of n-LAB. In addition, the long-term impact of prophylactic n-LAB use on the evolution of *Aspergillus* speciation and susceptibility to this drug was investigated.

In our LTR cohort, the overall incidence of *Aspergillus* spp. infection was 14.3% and IA incidence was 5.3%. Although it is difficult to compare *Aspergillus* spp. infection rates between studies, most publications in which universal or targeted *Aspergillus* spp. prophylaxis has been used in LTR have reported invasive disease rates ranging from 1.5% to 12.2% [17–19].

In terms of tolerability, our n-LAB prophylaxis strategy proved to be good [7,9,10] allowing lifetime maintenance.



Time/years	Person-time follow-up (days)	Rate	Incidence rate ratio	95% CI	P
2003–2008	214.53088	0.191	1.97	0.14-0.25	0.0003
2009–2014	857.06018	0.096	1.32	0.07-0.12	

Figure 2 Person-time incidence rates and incidence rate ratios of *Aspergillus* spp. infection and colonization, and evolution of *Aspergillus* spp. with reduced susceptibility or resistance (*A. flavus*, *A. terreus*, and *A. alliaceus*) to amphotericin from July 2003 to December 2008 (before 2009) and from January 2009 to July 2014 (after 2009). AB, amphotericin B.

Table 5. Risk factors potentially associated with the development of 59 *Aspergillus* spp. infections in 53 lung transplant patients.

Risk factors	*Aspergillus* spp. infections *n* (%)
Chronic gram-negative bacterial colonization	38/59 (64.4)
Bronchial stenosis without stent	28/59 (47.5)
Chronic lung allograft dysfunction	21/59 (35.6)
Acute rejection	15/59 (25.4)
CMV disease	14/59 (23.7)
Bronchial stent	14/59 (23.7)
Pre-transplant *Aspergillus* spp. colonization	9/59 (15.2)
Massive inhalation	8/59 (13.6)
Overimmunosuppression	6/59 (10.2)
Abandonment of prophylaxis	4/59 (6.8)
Induction immunosuppression	3/59 (5.1)

In our series, prophylaxis with n-LAB was well tolerated, with only 2.9% of adverse effects and 1.7% of patients requiring treatment withdrawal for this cause. Similar tolerance has been reported in other studies [8,9]. One disadvantage of this therapy is local irritation with secondary effects such as bronchospasm (1.9%), but the use of salbutamol or halving the drug concentration can improve these symptoms. Inhaled n-LAB has the advantage that distribution is limited to the respiratory tract, there is no systemic absorption [7,9], and high levels of antifungal concentrations can be achieved in the lung without changes in respiratory function. Thus, the risk of nephrotoxicity is averted and the drug can be administered over lengthy periods. In comparison with azoles, n-LAB has a lower incidence of systemic side effects (especially hepatotoxicity) [17,20,21], and an absence of interactions with immunosuppressive drugs and glucocorticoids [7].

Aspergillus spp. infection was formerly considered an immediate post-transplantation complication, but recent evidence indicates that it can occur much later after lung transplantation [2,15,22]. The high proportion of late-onset *Aspergillus* spp. infection (84.7%) cases and median time to the first *Aspergillus* spp. infection (266 days) in our cohort concur with these findings. Probably changes in routine antifungal prophylaxis patterns against *Aspergillus* species appear to be shifting the occurrence of *Aspergillus* spp. infection later after transplantation. Other factors that may led to higher rate of late-onset infections could be age, overimmunosuppression or changes in immunosuppressive regimens (sirolimus use in correlation with tacrolimus), and chronic lung allograft dysfunction [2,15,22].These results are of particular interest considering that the duration of antifungal prophylaxis is usually limited to the first 3–6 months after transplantation [3,23]. Unfortunately, a consensus does not exist on what length of treatment should be.

Aspergillus spp. colonization and infection rates have decreased in the last 5 years in our center. In contrast to the findings from a recent epidemiologic study on *Aspergillus* species in Spain [24,25] and previous series in LTR [1,2], significant increases have occurred in colonization and infection by *Aspergillus* species with reduced susceptibility or resistance to AB. However, these species did not seem to be associated with lower successful outcome or higher mortality in our series. These data may have different interpretations. Probably these results are related to a more extensive n-LAB use over time in daily practice [6–10]. As has been reported [7], our protocol prescribes n-LAB use every 2 weeks starting from 6 months post-transplantation. It may be that inhaled n-LAB concentrations at this lengthy dosing interval are not high enough to inhibit growth of these potentially resistant *Aspergillus* species, and this would favor late-onset colonization and infection. Moreover, primary *in vitro* resistance to AB has been observed for *A. terreus*, which is intrinsically resistant to AB and *A. flavus*, in which resistance is 10–15% [26]. In addition, resistance development during AB treatment is rare, but isolates recovered from patients who previously received AB have shown higher MICs than those from patients without AB exposure [5,27]. Although the current knowledge regarding emergence of resistant organisms in patients receiving prophylactic n-LAB is poor [5,27,28], this situation may be a sentinel event that needs to be monitored and strictly surveilled. Although delayed occurrence of Aspergillus infection in LTR has relevant implications, these worrisome long-term consequences of indefinite treatment and the controversial use of lifetime prophylaxis in LTR do not support the use of universal long- term prophylaxis and highlight the need of individualizing. Probably it may be advisable to recommend universal prophylaxis post-transplant (6–12 months) and consider an extended course, mainly in targeted high-risk patients (acute and chronic rejection, augmented immunosuppression and CMV infection, fungal colonization). In this situation, the convenient administration schedule of n-LAB (every 2 weeks) would be a positive factor, conducive to adherence [7].

Post-transplant *Aspergillus* spp. colonization is a known risk factor for subsequent IA in LTR [29]. The incidence of *Aspergillus* spp. colonization in LTR receiving various antifungals and no prophylaxis varies from 4% to 28.1% [3]. Of note, our colonization rate was 14.8%, all but 6 episodes resolved, and there was only one subsequent case of IA. These findings seem to indicate that our standard practice of increasing n-LAB dose to 3 times weekly when *Aspergillus* spp. colonization is detected suffices to prevent the development of IA.

Previous studies have suggested that CLAD may be a risk factor for subsequent *Aspergillus* spp. Infection [13,30]. Although we did not statistically control for other effects (as it was not the main aim of our study), the results of our investigation support this notion. Considering that LTR

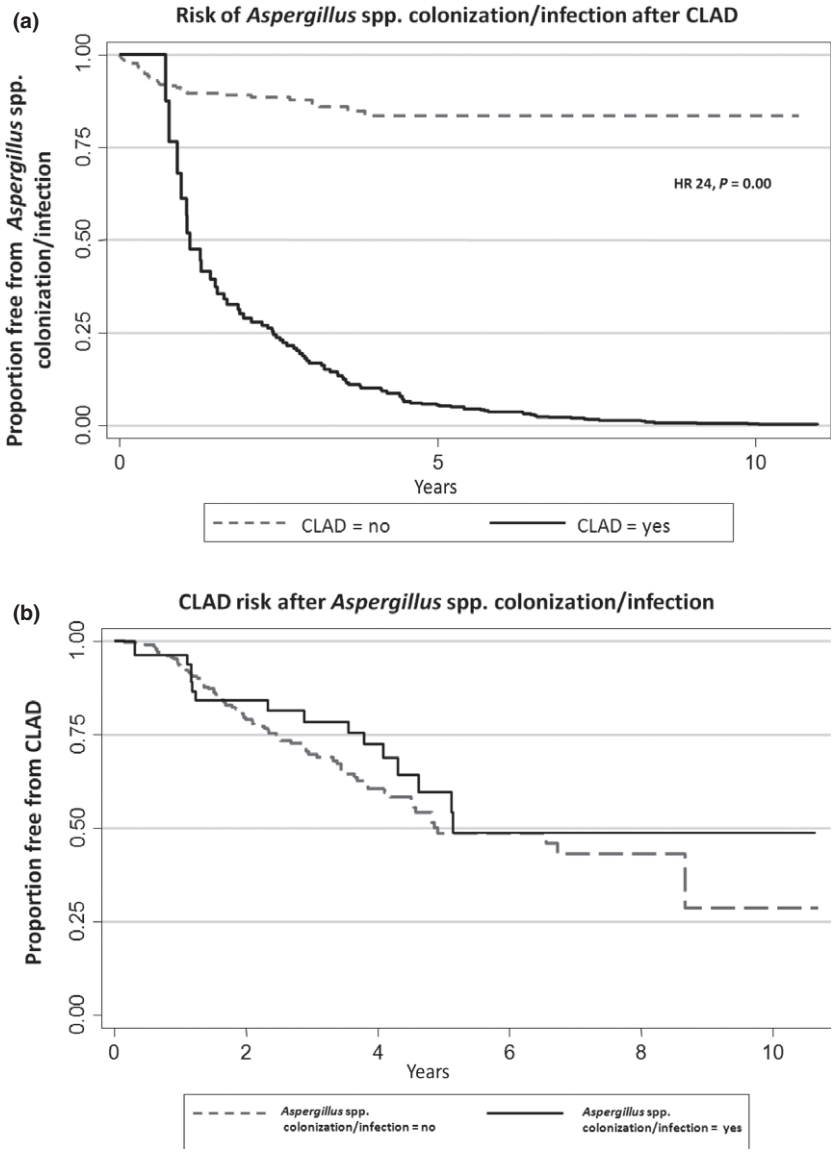


Figure 3 Time-dependent Cox-regression analysis: (a) development of *Aspergillus* spp. colonization or infection after chronic lung allograft dysfunction (CLAD); (b) Development of CLAD after *Aspergillus* spp. colonization or infection.

with CLAD may have a combination of overimmunosuppression and nonuniformly distributed restrictive and obstructive processes that can affect regional n-LAB deposition and favor *Aspergillus* spp. infection [31], it could be reasonable to intensify the frequency of n-LAB administration in these patients. Nonetheless, although previous studies have found an association between *Aspergillus* spp. colonization and infection [32,33] and posterior development of CLAD, our results did not confirm this relationship.

Although previous reports have alluded to a higher incidence of *Aspergillus* spp. infections in single lung transplant recipients [34], this difference was not observed in our cohort and the incidence of aspergilloma in native lung was low. It is likely that n-AB distribution occurs preferentially in the allograft, with unreliable distribution in the native lung, although sufficient to prevent *Aspergillus* spp. infection [31].

Of interest, chronic colonization by gram-negative bacteria in our series was often associated (57.4%) with *Aspergillus* spp. infection. The reason for this association is uncertain. It may be that LTR with CLAD may have a parenchymal disease that could advantage proliferation of multiple organisms. Moreover, the local lung milieu in

patients colonized by gram-negative bacteria could favor proliferation of *Aspergillus* spp., as has been described in cystic fibrosis patients [13,35].

Bronchial stent infections have a poor prognosis, with very low cure rates. The presence of a foreign body, which can act as a fungal reservoir, may promote *Aspergillus* spp. biofilm formation, making antifungal penetration difficult [36].

The main limitation of this study is its observational and retrospective design and the necessary assumption of changes in the diagnosis and therapy of *Aspergillus* spp. infection to adapt to advances in the management of these patients over the lengthy study period. Lastly, in our cohort, incidence of cystic fibrosis (6.3%) is lower than reported in other series [3]. Therefore, it may need to be taken into account when comparing *Aspergillus* spp. infection and colonization rates with other studies, because it may be underestimated.

In conclusion, prophylaxis with n-LAB at the dose and frequency described has proved to be tolerable and can be used for preventing *Aspergillus* spp. infection in LTR. Over the last years, the incidence of *Aspergillus* spp. colonization and infection has decreased. Nevertheless, *Aspergillus* species with reduced susceptibility or resistance to AB are emerging but do not seem to be associated with lower successful outcome or higher mortality in our series. Emergence of resistant organisms in patients receiving prophylactic n-LAB may be a sentinel event that needs surveillance. CLAD is associated with the development of *Aspergillus* spp. colonization and infection, and n-LAB prophylaxis could be intensified in patients with this factor. A multicenter randomized controlled trial is warranted to assess the efficacy of *Aspergillus* spp. prophylaxis in LTR.

Authorship

MP: participated in the research design, in the performance of the research, in data analysis, and in the writing of the paper. VM: participated in the research design, in the performance of the research, in data analysis, and in the writing of the paper. MTM-G: participated in the performance of the research and in data collection and commented on the final version of the manuscript. IR-C: participated in the interpretation of the results, supervised the testing, and commented on the final version of the manuscript. CB: participated in the performance of the research and interpretation of the results and approved the final version of the manuscript. BS: participated in the performance of the research and interpretation of the results and approved the final version of the manuscript. JR: participated in the performance of the research and interpretation of the results and approved the final version of the manuscript. PU: participated in the interpretation of the results and commented on the final version of the manuscript. JS: participated in the interpretation of the results and commented on the final version of the manuscript. JG: participated in the interpretation of the results and approved the final version of the manuscript. AR: participated in the interpretation of the results, supervised the testing, and commented on the final version of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplemental material and methods

Figure S1. *Aspergillus* spp. infections and colonization.

References

1. Pappas PG, Alexander BD, Andes DR, *et al.* Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). *Clin Infect Dis* 2010; **50**: 1101.
2. Neofytos D, Fishman JA, Horn D, *et al.* Epidemiology and outcome of invasive fungal infections in solid organ transplant recipients. *Transpl Infect Dis* 2010; **12**: 220.
3. Bhaskaran A, Mumtaz K, Husain S. Anti-aspergillus prophylaxis in lung transplantation: a systematic review and meta-analysis. *Curr Infect Dis Rep* 2013; **15**: 514.
4. Monforte V, Roman A, Gavalda J, *et al.* Nebulized amphotericin B prophylaxis for *Aspergillus* infection in lung transplantation: study of risk factors. *J Heart Lung Transplant* 2001; **20**: 1274.
5. Lionakis MS, Lewis RE, Torres HA, Albert ND, Raad II, Kontoyiannis DP. Increased frequency of non-fumigatus *Aspergillus* species in amphotericin B- or triazole-pre-exposed cancer patients with positive cultures for aspergilli. *Diagn Microbiol Infect Dis* 2005; **52**: 15.

6. Monforte V, Ussetti P, Gavalda J, *et al.* Feasibility, tolerability, and outcomes of nebulized liposomal amphotericin B for *Aspergillus* infection prevention in lung transplantation. *J Heart Lung Transplant* 2010; **29**: 523.
7. Monforte V, Ussetti P, Lopez R, *et al.* Nebulized liposomal amphotericin B prophylaxis for *Aspergillus* infection in lung transplantation: pharmacokinetics and safety. *J Heart Lung Transplant* 2009; **28**: 170.
8. Drew RH, Dodds Ashley E, Benjamin DK Jr, Duane Davis R,Palmer SM, Perfect JR. Comparative safety of amphotericin B lipid complex and amphotericin B deoxycholate as aerosolized antifungal prophylaxis in lung-transplant recipients. *Transplantation* 2004; **77**: 232.
9. Lowry CM, Marty FM, Vargas SO, *et al.* Safety of aerosolized liposomal versus deoxycholate amphotericin B formulations for prevention of invasive fungal infections following lung transplantation: a retrospective study. *Transpl Infect Dis* 2007; **9**: 121.
10. Monforte V, Lopez-Sanchez A, Zurbano F, *et al.* Prophylaxis with nebulized liposomal amphotericin B for *Aspergillus* infection in lung transplant patients does not cause changes in the lipid content of pulmonary surfactant. *J Heart Lung Transplant* 2013; **32**: 313.
11. De Pauw B, Walsh TJ, Donnelly JP, *et al.* Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin Infect Dis* 2008; **46**: 1813.
12. Husain S, Mooney ML, Danziger-Isakov L, *et al.* A 2010 working formulation for the standardization of definitions of infections in cardiothoracic transplant recipients. *J Heart Lung Transplant* 2011; **30**: 361.
13. Valentine VG, Bonvillain RW, Gupta MR, *et al.* Infections in lung allograft recipients: ganciclovir era. *J Heart Lung Transplant* 2008; **27**: 528.
14. Singh N, Husain S, Practice ASTIDCo. Aspergillosis in solid organ transplantation. *Am J Transplant* 2013;**13** (Suppl 4):228.
15. Gavalda J, Len O, San Juan R, *et al.* Risk factors for invasive aspergillosis in solid-organ transplant recipients: a case-control study. *Clin Infect Dis* 2005; **41**: 52.
16. Herbrecht R, Denning DW, Patterson TF, *et al.* Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N Engl J Med* 2002; **347**: 408.
17. Husain S, Paterson DL, Studer S, *et al.* Voriconazole prophylaxis in lung transplant recipients. *Am J Transplant* 2006; **6**: 3008.
18. Eriksson M, Lemstrom K, Suojaranta-Ylinen R, *et al.* Control of early *Aspergillus* mortality after lung transplantation: outcome and risk factors. *Transpl Proc* 2010; **42**: 4459.
19. Hayes D Jr, Ball AM, Mansour HM, Martin CA, Flynn JD. Fungal infection in heart-lung transplant recipients receiving single-agent prophylaxis with itraconazole. *Exp Clin Transplant* 2011; **9**: 399.
20. Cadena J, Levine DJ, Angel LF, *et al.* Antifungal prophylaxis with voriconazole or itraconazole in lung transplant recipients: hepatotoxicity and effectiveness. *Am J Transplant* 2009; **9**: 2085–91.
21. Mitsani D, Nguyen MH, Shields RK, *et al.* Prospective, observational study of voriconazole therapeutic drug monitoring among lung transplant recipients receiving prophylaxis: factors impacting levels of and associations between serum troughs, efficacy, and toxicity. *Antimicrob Agents Chemother* 2012; **56**: 2371.
22. Singh N, Limaye AP, Forrest G, *et al.* Late-onset invasive aspergillosis in organ transplant recipients in the current era. *Med Mycol* 2006; **44**: 445.
23. Neoh CF, Snell GI, Kotsimbos T, *et al.* Antifungal prophylaxis in lung transplantation–a world-wide survey. *Am J Transplant* 2011; **11**: 361.
24. Alastruey-Izquierdo A, Mellado E, Pelaez T, *et al.* Population-based survey of filamentous fungi and antifungal resistance in Spain (FILPOP Study). *Antimicrob Agents Chemother* 2013; **57**: 3380.
25. Garcia-Vidal C, Royo-Cebrecos C, Peghin M, *et al.* Environmental variables associated with an increased risk of invasive aspergillosis. *Clin Microbiol Infect* 2014; **20**: O939.
26. Alastruey-Izquierdo A, Gomez-Lopez A, Arendrup MC, *et al.* Comparison of dimethyl sulfoxide and water as solvents for echinocandin susceptibility testing by the EUCAST methodology. *J Clin Microbiol* 2012; **50**: 2509.
27. Torres HA, Rivero GA, Lewis RE, Hachem R, Raad II, Kontoyiannis DP. Aspergillosis caused by non-fumigatus *Aspergillus* species: risk factors and in vitro susceptibility compared with *Aspergillus fumigatus*. *Diagn Microbiol Infect Dis* 2003; **46**: 25.
28. Caston JJ, Linares MJ, Gallego C, *et al.* Risk factors for pulmonary *Aspergillus terreus* infection in patients with positive culture for filamentous fungi. *Chest* 2007; **131**: 230.
29. Cahill BC, Hibbs JR, Savik K, *et al. Aspergillus* airway colonization and invasive disease after lung transplantation. *Chest* 1997; **112**: 1160.
30. Sole A, Morant P, Salavert M, Peman J, Morales P, Valencia Lung Transplant Group. *Aspergillus* infections in lung transplant recipients: risk factors and outcome. *Clin Microbiol Infect*. 2005;**11**:359.
31. Monforte V, Roman A, Gavalda J, *et al.* Nebulized amphotericin B concentration and distribution in the respiratory tract of lung-transplanted patients. *Transplantation* 2003; **75**: 1571–4.
32. Weigt SS, Elashoff RM, Huang C, *et al. Aspergillus* colonization of the lung allograft is a risk factor for bronchiolitis obliterans syndrome. *Am J Transplant* 2009; **9**: 1903.
33. Weigt SS, Copeland CA, Derhovanessian A, *et al.* Colonization with small conidia *Aspergillus* species is associated with bronchiolitis obliterans syndrome: a two-center validation study. *Am J Transplant* 2013; **13**: 919–27.

34. Westney GE, Kesten S, De Hoyos A, Chapparro C, Winton T, Maurer JR. *Aspergillus* infection in single and double lung transplant recipients. *Transplantation* 1996; **61**: 915.
35. Paugam A, Baixench MT, Demazes-Dufeu N, *et al.* Characteristics and consequences of airway colonization by filamentous fungi in 201 adult patients with cystic fibrosis in France. *Med Mycol* 2010; **48**(Suppl 1): S32.
36. Muller FM, Seidler M, Beauvais A. *Aspergillus* fumigatus biofilms in the clinical setting. *Med Mycol* 2011; **49**(Suppl 1): S96.