Review

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How should we approach *Aspergillus* in lung secretions of patients with COPD?

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ABSTRACT

Aspergillus spp. is frequently isolated in respiratory samples from patients with severe COPD; however, the clinical significance of this mold is unclear and its presence may indicate temporary passage, benign chronic carriage, or onset of invasive disease. The definitive diagnosis of pulmonary aspergillosis in COPD patients is often difficult owing to the lack of specific clinical and radiological signs. However, retrospective studies show the risk for developing pulmonary aspergillosis in older patients with severe COPD, and a high number of comorbidities who have received treatment with corticosteroids and/or broad spectrum antibioties. The development of algorithms based on microbiological and radiological data and risk factors for pulmonary aspergillosis can help to differentiate between colonization and infection.

Key words: *Aspergillus*, chronic pulmonary obstructive disease, voriconazole, liposomal amphotericin B

¿Qué hacer ante el hallazgo de *Aspergillus* en secreciones pulmonares de pacientes con EPOC?

RESUMEN

El aislamiento de *Aspergillus* spp. en muestras respiratorias en frecuente actualmente en la EPOC avanzada, pero su significado clínico es incierto, pudiendo representar un estado temporal, un portador crónico o un signo que precede a la infección. El diagnóstico definitivo de la aspergilosis pulmonar en los pacientes con EPOC es a veces difícil de debido a la falta de signos clínicos y radiológicos específicos. Sin embargo, el riesgo de aspergilosis pulmonar es bien conocido, a través de estudios retrospectivos, en pacientes con EPOC grave, de edad avanzada, con gran número de comorbilidades y tratamientos previos con corticoides y/o antibióticos de amplio espectro. El desarrollo de algoritmos basados en datos clínicos y radiológicos y en factores de riesgo para la aspergilosis pulmonar puede ayudar a diferenciar la colonización de la infección.

Palabras clave: *Aspergillus*, enfermedad pulmonar obstructiva crónica, voriconazol, anfotericina B liposómica

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INTRODUCTION

Acute pulmonary aspergillosis (APA) is no longer limited to bone marrow recipients[1] and is now a common finding in other types of patient, eg, those with structural lung disease, particularly chronic obstructive pulmonary disease (COPD)[2].

Isolation of *Aspergillus* spp. in the respiratory tract of patients with hematologic diseases has been associated with a proven, probable, or possible diagnosis of APA depending on host characteristics, clinical manifestations, and type of positive sample[3]. A similar approach has been attempted in patients with non-neutropenic disease[4] (table 1), although the clinical significance of lung colonization by *Aspergillus* remains uncertain and may represent a simple temporary state, chronic carriage, or onset of invasive infection[4-7].

PATHOGENESIS

Inhalation of *Aspergillus* conidia is common, and it is thought that we inhale >200 per day. The conidia with the smallest diameter reach the deepest parts of the bronchial tree, and therefore *Aspergillus fumigatus* is the species most commonly found in the airway. Nevertheless, the risk of infection is minimal, provided that the respiratory tract remains anatomically and functionally sound, with no impairment of

Table 1 **Definition of acute pulmonary aspergillosis in patients with chronic obstructive pulmonary disease[4].**

Type of APA	Criteria
Proven	Histology or cytology examination by fine needle aspiration or biopsy of any pulmonary lesion over a 3-month period that reveals hyphae compatible with *Aspergillus* and evidence of tissue damage if accompanied by any of the following findings: 1) Positive culture for *Aspergillus* from any lower respiratory tract sample; 2) Positive serology results for *A. fumigatus*; 3) Confirmation that the hyphae observed are *Aspergillus* using molecular biology techniques, immunology-based methods, or culture.
Probable	Same as proven APA, although with no confirmation that *Aspergillus* is the causal agent (points 1, 2, and 3 not present or tested for) or advanced COPD (GOLD III or IV) in a patient who is usually treated with corticosteroids and has experienced a recent exacerbation of dyspnea[a], chest x-ray or CT scan showing compatible images[b] within <3 months[c], and one of the following situations: 1) Microscopic evidence or positive culture[d] of *Aspergillus* in a lower respiratory tract sample 2) Positive serology result for *A. fumigatus* 3) Two positive results in determination of serum galactomannan
Possible	Patient with advanced COPD (GOLD III or IV) habitually treated with corticosteroids, with recent exacerbation of dyspnea[a], chest x-ray or CT scan with compatible images[b] within <3 months[c], although with negative results for *Aspergillus* in serology testing or culture
Colonization	Positive *Aspergillus* culture in lower respiratory tract sample with no exacerbation of dyspnea or new pulmonary infiltrate

APA: acute pulmonary aspergillosis

GOLD: Global Initiative for Chronic Obstructive Lung Disease

CT: computed tomography

[a]**Exacerbation of dyspnea or bronchospasm resistant to appropriate treatment including antibiotics.**

[b]**Pulmonary lesion with no response to treatment with an appropriate antibiotic (dose, route, spectrum, and activity).**

[c]**Especially if the image reveals cavitary lesions. After 3 months these are considered chronic pulmonary aspergillosis, unless direct tissue invasion can be demonstrated.**

[d]**Standard culture, culture in Sabouraud glucose agar, or detection using molecular techniques.**

the immune response, especially at the level of alveolar macrophages[8]. Maintenance of airway sterility essentially depends on the rate of bronchial drainage and the concentration of antibiotics in bronchial secretions[8,9].

The respiratory tract epithelium of patients with COPD is changed to different degrees, mainly in the bronchioles. The changes observed include the following: chronic inflammatory infiltration of the mucosa (induced mainly by macrophages and CD8+ lymphocytes); reduced number and length of ciliary cells and hypertrophy of the bronchial submucosal glands and goblet cells (which lead to overproduction of mucus and fibrosis of the airway); and slow and irreversible bronchial obstruction and reduced efficacy of the mucociliary drainage system that enable *Aspergillus* conidia to be trapped and the bronchial lumen to be colonized. Conidia that have not been cleared from the bronchial lumen interact with a series of components: 1) soluble lung components (eg, collectins, lysozyme, C3, pentraxin-3, plasminogen, and cationic peptides), which agglutinate conidia and prepare them for phagocytosis; 2) the bronchial epithelium itself, especially when damaged; and 3) alveolar macrophages (more distally)[8]. Alveolar macrophages are the first line of the innate immune response to inhaled conidia. They recognize and bind to the mold mainly via 2 types of transmembrane pattern recognition receptors (PRR), namely, toll-like receptors (TLR) 2 and 4, and dectin-1. Binding of conidia to PRRs activates phagocytosis and opsonization and implements the acquired immune response through expression of various cytokines. TLR-2 express IL-4 and IL-10, leading to a Th2 lymphocyte–mediated inflammatory response in allergic reactions. In contrast, by expressing IL-1, IL-12, IL-15, and interferon alfa and gamma, TLR-4 trigger a protective Th1 lymphocyte–mediated proinflammatory response to infection[10,11]. The proinflammatory activity of dectin-1 is similar to that of TLR-2; in addition, dectin-1 stimulates activation of intracellular NADPH-oxidase[10,11]. Phagosomes are responsible for destruction of conidia inside the cell, where acidification seems to play a major role[12]. Neutrophils eliminate the hyphae produced by the conidia, a process involving the intervention of NADPH-oxidase and other oxidative mechanisms generated from intracellular granules[10,11]. Surviving hyphae can cross the alveolar-capillary barrier, and some fragments are released into the bloodstream, through which they are disseminated to distant organs if dendritic cells, monocytes, and neutrophils are not sufficient in number or are not competent[13].

In addition to their predisposition to colonization, patients with COPD also present well-known risk factors that favor lung infection by *Aspergillus* spp., such as previous broad-spectrum antibiotic therapy and treatment with corticosteroids[8,14-16].

CLINICAL MANIFESTATIONS

COPD is frequently present in patients with subacute pulmonary aspergillosis and chronic pulmonary aspergillosis and seems to play a role in the development of both conditions[15,17]. However, since the beginning of the century, the frequency of APA has been increasing considerably in patients with GOLD grade ≥II COPD who are being or have been treated with

Table 2 Applicable microbiological techniques according to type of sample and necessary volume

Technique - volume	Sputum/BAS	BAL	Blood/Serum	Biopsy	Pleural fluid
Culture and microscopy - 1 mL	Yes	Yes	No	Yes	Yes
ß-D-Glucan - 200 µL	No	Yes	Yes	No	No
Galactomannan - 600 µL	SI	Yes	Yes	Yes	Yes
PCR - 2 mL	SI	Yes	Yes	Yes	Yes
LFD - 100 µL	No	Yes	Yes	No	No

BAS: bronchial aspirate; BAL: bronchoalveolar lavage; PCR: polymerase chain reaction; LFD: lateral flow device.

corticosteroids[2,16,18-24]. Today, more than 10% of cases of APA are seen in patients with COPD, and this figure seems to be increasing[16,18,19,22-26]. Similarly, the mortality of APA is high in COPD patients, even more so than in patients with hematologic diseases, and seems to be associated with delay in diagnosis and initiation of treatment[2,4,23,25].

In patients with COPD, chronic forms of pulmonary aspergillosis are easily recognized based on clinical and radiological evidence. Aspergilloma is usually asymptomatic and can sometimes trigger hemoptysis. Radiology reveals a mobile image inside pre-existing lung cavities[27]. Chronic necrotizing aspergillosis is an indolent process that sometimes produces fever, cough, and expectoration. The most common radiological image reveals localized cavitations in the apex of the lung, with frequent pleural involvement[28]. APA shows no specific clinical manifestations in COPD that enable it to be identified definitively (eg, fever, dyspnea, cough, expectoration, hemoptysis, and impaired general condition). However, it should be suspected in patients with advanced disease and localized manifestations (infiltrates, nodules, and cavitations) that do not respond to appropriate antibiotic therapy[4].

DIAGNOSIS

In clinical terms, there are no specific signs or symptoms of APA in patients with COPD[4,26,29]. The same is true of radiological images, and the halo sign is not common[2,4,29]. Nevertheless, infiltrates, nodules, and cavitations have been shown to be significantly more frequent in APA than in colonization[2].

As for microbiological diagnosis, the several laboratory tools available range from classic microscopy and culture to novel immunochromatographic tests (table 2)[30]. In general terms, all the available techniques—with the exception of glucan determination—offer better results in respiratory samples than in blood. Clinical samples must be taken to demonstrate the presence of the mold, whether by isolation using culture media or by indirect demonstration of fungal components, mainly fungal DNA or cell wall antigens. However, in patients with advanced COPD, it is not easy to obtain respiratory samples using invasive procedures, and sputum is the only available sample in most cases[4]. The classic technique consists of the culture of respiratory samples, sputum, bronchial aspirate (BAS), and bronchoalveolar lavage (BAL) fluid in Sabouraud agar with gentamicin and chloramphenicol, which is incubated at 35ºC. If the sample analyzed contains *Aspergillus* under these conditions, growth of characteristic colonies will occur at 24-48 hours, thus enabling identification of the genus and species in most cases. In addition, a rapid presumptive diagnosis can be made before culture by observing a fresh sample under the light microscope or with calcofluor-white stain in the case of the fluorescence microscope. We can observe "*Aspergillus*-compatible" septate hyphae, although, as in any stain or histopathology slice, we will never be able to specify with any degree of certainty the genus of the fungus by microscopy only, since septate hyphae are found in all hyaline molds, including other hyalohyphomycetes such as *Fusarium* or *Scedosporium*. The main advantage of culture is its low cost. In addition, despite its low sensitivity (30%-35% at most), specificity can reach 95%, thus making it possible to isolate the mold for *in vitro* sensitivity testing. At the same time, culture makes it possible to grow other types of mold in infections other than aspergillosis, ie, those caused by *Fusarium*, *Scedosporium*, or a zygomycete in the case of mucormycosis. The probability of APA has been shown to increase with the number of positive respiratory samples[31].

As stated above, the development of molecular techniques has increased our knowledge of the taxonomy of *Aspergillus* species. Thus, we can now differentiate between the most common species and between species and varieties that are genetically different but phenotypically indistinguishable. Such is the case in all species of *Aspergillus* known to be involved in human disease, such as *A. fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus nidulans*. For example, *A. fumigatus* complex contains different sections or microsatellites that are expressed asexually or anamorphically as *Aspergillus* and sexually or teleomorphically as *Neosartorya*. Within the fumigati section, we can differentiate between entities such as *A. fumigatus* "senso stricto", *Aspergillus lentulus*, *Aspergillus novofumigatus*, and *Aspergillus viridinutans*[32]. Species from the complex that are expressed as *Neosartorya* are characterized by the development of more aggressive and prolonged clinical pictures and present lower susceptibility to antifungals (both azoles and amphotericin B)[33]. Identification of all the varieties contained within the genus *Aspergillus* is not currently feasible for a hospital clinical

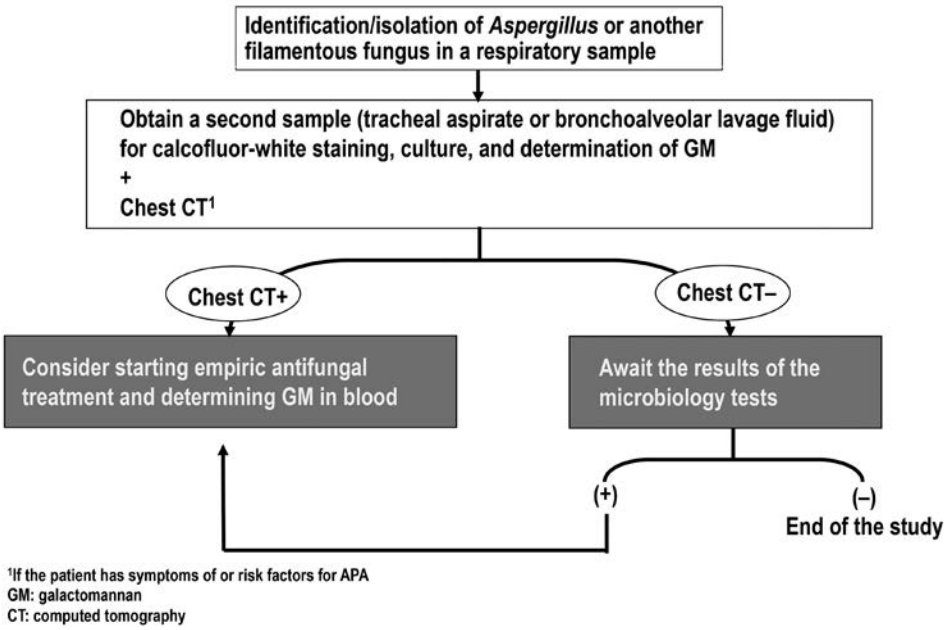


Figure 1 | **Diagnostic procedure**

microbiology laboratory, although some hospitals have technology that complements culture and for which broad experience has been garnered in other fields, such as bacteriology. The technology in question is laser spectrophotometry, known commercially as MALDITOF® (Bruker), which has been developed over the last 10 years and also covers fungi, including *Aspergillus*. MALDITOF is as reliable as polymerase chain reaction (PCR), although the database of the software should be extended so that the protein spectrum obtained after laser-assisted lysis of the mold coincides with reported data[34-36].

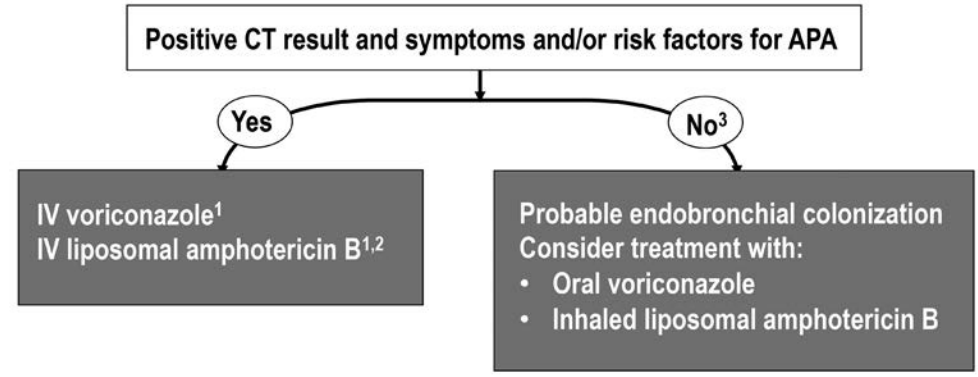
As stated above, an undeniable advantage of culture is the possibility it offers to perform antifungal susceptibility testing. The two standardized procedures for determining antifungal susceptibility are those developed by EUCAST (*European Committee of Antimicrobial Susceptibility Testing*) and the CLSI (*Clinical and Laboratory Standards Institute*)[37]. Both provide cut-off points, mainly for *Candida* spp., to classify isolates as susceptible or resistant. With respect to *Aspergillus*, the techniques are hampered by the fact that many of the cut-off points are either not defined or based on epidemiological recommendations. Broth microdilution continues to be laborious and has been replaced by techniques whose results show good correlations such as the E-test® (AB Biodisk), which consists of a cellulose strip impregnated with increasing concentrations of antifungal on an agar plate inoculated with a specific concentration of *Aspergillus* conidia. Despite being simpler than agar dilution, it must be performed in a biosafety cabinet to avoid contamination, and the staff performing it must be fully trained. The results must be assessed by an expert microbiologist, since they are sometimes difficult to interpret and may be invalid, with little or no clinical applicability.

Although it has proven useful in other types of mycosis, blood culture should not be performed in cases of clinically suspected aspergillosis, given that the whole mold does cross the vascular endothelium and is not found in sufficiently viable form in the bloodstream to enable its growth in culture media. We can only find some wall components (see below) in cases of angioinvasion and dissemination in advanced stages of infection.

In addition to direct diagnosis using culture, we have at our disposal a series of indirect techniques for detection of molds. We can determine cell wall antigens, such as β-D-glucan and galactomannan, and fungal DNA using molecular techniques such as PCR[37]. A number of new techniques have appeared in recent months. These include the so-called lateral flow device (LFD), which uses a monoclonal antibody (JF5) in an immunochromatographic support to detect a glycoprotein secreted by *Aspergillus* during multiplication[38].

The techniques that are best known and within the reach of most microbiology laboratories are enzyme-linked immunosorbent assay (ELISA), which is used to detect the main antigens in the *Aspergillus* wall. Since glucan is a "panfungal" biomarker, almost all fungi have glucan in their wall, with the result that the positive predictive value of the test varies from 40% to 50%. Positive results can also be detected in a patient with candidiasis or *Pneumocystis jiroveci* pneumonia. In contrast, its negative predictive value, that is, its power to reliably rule out fungal infection, reaches 95%, except in mucormycosis and cryptococcosis, since the walls of these fungi do not contain glucan.

As for galactomannan, it is important to remark that this component of the *Aspergillus* cell wall is released during



[1]If disease course is not favorable, consider:
- Combination with antifungal agents
- Combination with inhaled liposomal amphotericin B

[2]In the case of:
- A mold other than *Aspergillus*
- Treatment with drugs that prolong the QT interval (macrolides, metronidazole, pentamidine, foscarnet, citalopram, fluoxetine, nortriptyline, ondansetron, granisetron)
- Liver disease (≥5 times normal transaminase values)

[3]At least 2 positive respiratory samples

Figure 2 | **Empiric treatment**

growth of the mold during tissue invasion and that if there is no infection, galactomannan will not be detected in the sample. This observation is particularly relevant in respiratory samples, where it is important to distinguish between colonization and infection. The reliability of determination of galactomannan in neutropenic patients is widely reported in the literature[39]. Current scientific evidence in non-neutropenic patients supports the use of galactomannan, with two conditions: BAL fluid—not blood—must be the sample of choice, and the cut-off must be raised from 0.5 ng/mL to 0.8 ng/mL or even 2.0 ng/mL according to some authors[40,41]. The sensitivity of the test for these cases exceeds 70%, and the specificity has been reported to reach 98%[42]. At present, galactomannan in BAL is considered the gold standard for diagnosis of pulmonary aspergillosis in non-neutropenic patients, and a positive result is also a prognostic factor. Serum galactomannan is less sensitive in non-neutropenic than in neutropenic patients, perhaps because, in non-neutropenic patients, aspergillosis presents as a more subacute or insidious condition, with reduced passage of fungal components to blood.

Yet another diagnostic tool at our disposal is detection of fungal DNA, which must be amplified using PCR. No standardized and reproducible PCR technique is currently available for universal use in microbiology laboratories; therefore, given the expense and complexity of the technique, it is sometimes customized using in-house primers. PCR is a very sensitive technique that can detect up to 1-10 pg of DNA, that is, infinitely small quantities of mold (10-100 conidia per sample)[43]. Therefore, it is easily contaminated with environmental conidia and can yield false-positive results. This risk can be minimized if the technique is performed in a biosafety cabinet under aseptic conditions by trained personnel. As for interpretation, it is important to remember that when we analyze a patient's blood, we are detecting DNAemia, not fungemia, and that the DNA could correspond to a nonviable or phagocytosed fungus.

Lastly, new techniques that are faster and simpler to perform than current techniques are emerging. These include the immunochromatographic LFD, which can detect *Aspergillus*-specific protein in as little as 10 minutes. This highly specific technique (92%-98%) uses a monoclonal antibody and therefore does not present cross-reactions with other molds. However, despite the apparent simplicity of the technique, the results must be interpreted by a microbiologist acquainted with this type of "all-or-nothing" qualitative test; the subjectivity of a nonexpert observer could lead to false-positive results. Although the technique is not yet marketed in Spain, has been reported to represent a huge advance in the microbiological diagnosis of aspergillosis, both in neutropenic patients and in non-neutropenic patients[42].

All in all, the possibilities for microbiological diagnosis of aspergillosis seem to be expanding, and the most profitable strategy, as advocated in the literature, is that of combining several techniques simultaneously, bearing in mind that infection by *Aspergillus* is a dynamic process: galactomannan and other components are released during active multiplication of the fungus, and fungal DNA is released into the bloodstream during angioinvasion and dissemination. This does not seem to be the case in non-neutropenic patients. Combination of several techniques, which should always include culture because of its price and accessibility, and some of the others mentioned above (generally galactomannan in BAL fluid, with[out] PCR),

will enable us to achieve, as shown in the literature, optimal results, with a sensitivity of 100% and specificity of 98%, even in non-neutropenic patients[42-45]. When LFD becomes available, it should be included in the diagnostic armamentarium in order to improve the speed at which the microbiology laboratory can work. This is a key aspect in guaranteeing appropriate treatment for patients with aspergillosis.

MANAGEMENT

Unfortunately, despite all the improvements made in the diagnosis of APA in patients with COPD[2,4,16,24,25,46], the reality is that the clinical significance of isolating *Aspergillus* conidia in respiratory samples remains unclear. However, a passive attitude could increase the risk of death. Therefore, once the fungus is detected in a respiratory sample, the decision to start empiric antifungal treatment should be based on the patient's current clinical status and on the presence or absence of risk factors for the development of APA. These include previous or current treatment with corticosteroids or antimicrobials, advanced COPD (GOLD≥III), pulmonary cavitation, radiological and clinical deterioration, high comorbidity, and stay in the ICU[2,16,25]. Specific algorithms have already been designed for critically ill and non–critically ill patients[47-49].

Aspergillus should be investigated in respiratory secretions from patients with COPD who are at risk of APA. If the mold is isolated, confirmation should be sought in additional respiratory samples using staining, culture, and galactomannan. Where possible, these samples should be more reliable than sputum (eg, BAS or BAL fluid). Similarly, it would be appropriate to perform a CT scan of the chest if risk factors for or clinical suspicion of APA are present (figure 1)[49]. If the CT scan reveals compatible lesions (nodules, infiltrates, or cavitations) and the patient has not progressed satisfactorily with appropriate antibiotic therapy, empiric antifungal therapy and determination of galactomannan in blood should be considered. If the CT scan of the chest is negative, it is advisable to wait for the results of the microbiology tests before deciding whether or not to treat (figure 1).

Antifungal treatment can consist of intravenous voriconazole or liposomal amphotericin B[48]. Liposomal amphotericin B is particularly indicated when a mold other than *Aspergillus* is isolated or when voriconazole cannot be prescribed because of liver toxicity or interference with the metabolism of drugs that prolong the QT interval. If the response to the initial treatment is not good, both drugs can be combined or liposomal amphotericin B can be added as inhaled therapy in both cases, although this indication is not well established (figure 2)[48,50,51].

Negative results in the second respiratory samples and the CT scan probably indicate colonization or contamination of the first sample that has no clinical significance and does not require treatment. Nevertheless, in cases of colonization, if there is a risk of developing APA and the patient needs to take corticosteroids, prophylaxis with oral voriconazole or inhaled liposomal amphotericin B should be administered (figure 2)[50]. It is important to determine whether patients with COPD who are continuously colonized with *Aspergillus* need antifungal treatment to reduce the *Aspergillus* load during acute exacerbations, in much the same way as patients who are prescribed antibiotics for exacerbations.

The appropriate duration of treatment of APA in COPD patients—as in patients with hematologic diseases—has not been clearly defined, although it should be maintained until symptoms resolve and radiologic improvement has been observed. The high bioavailability of oral voriconazole means that sequential therapy with this regimen facilitates prolongation of treatment (both in APA and in chronic pulmonary aspergillosis), reduces hospital stay, and improves the patient's quality of life[52]. Surgical resection can be considered in localized forms of the disease with a poor response or intolerance to treatment, providing that functional residual capacity is acceptable[53].

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