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CHALLENGES IN THE DIAGNOSIS AND TREATMENT OF MUCORMYCOSIS

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ABSTRACT
The diagnosis and treatment of mucormycosis are challenging. The incidence of the disease seems to be increasing. Hematological malignancies are the most common underlying disease in countries with high income and uncontrolled diabetes in developing countries. Clinical approach to diagnosis lacks sensitivity and specificity. Radiologically, multiple (≥10) nodules and pleural effusion are reportedly associated with pulmonary mucormycosis. Another finding on computerized tomography (CT) scan, which seems to indicate the presence of mucormycosis, is the reverse halo sign. Microscopy (direct and on histopathology) and culture are the cornerstones of diagnosis. Molecular assays can be used either for detection or identification of mucormycetes, and they can be recommended as valuable add-on tools that complement conventional diagnostic procedures. Successful management of mucormycosis is based on a multimodal approach, including reversal or discontinuation of underlying predisposing factors, early administration of active antifungal agents at optimal doses, complete removal of all infected tissues, and use of various adjunctive therapies.Our armamentarium of antifungals is slightly enriched by the addition of two newer azoles (posaconazole and isavuconazole) to liposomal amphotericin B, which remains the drug of choice for the initial antifungal treatment, according to the recently published guidelines by ECIL-6, as well as those published by ECMM/ESCMID. Despite the efforts for better understanding of the pathogenesis, early diagnosis and aggressive treatment of mucormycosis, the mortality rate of the disease remains high.

INTRODUCTION
Mucormycosis is a rare, emerging fungal Infection, with high morbidity and mortality.Mucormycetes belong to the order Mucorales, subphylum Mucoromycotina.[1] Due to the rarity of the disease, it is almost impossible to conduct large, randomized clinical trials, and most of the available data regarding epidemiology, diagnosis, and treatment, originate from case reports and case series.

The mortality of mucormycosis remains high.Treatment includes antifungal agents in combination with surgical intervention. The only new agent with activity against Mucorales is isavuconazole, but it does not seem to offer significant advantages over historical first line therapy of amphotericin B-based drugs or posaconazole. The aim of many researchers is to find new methods for making the diagnosis of mucormycosis earlier, as early diagnosis of mucormycosis leads to improved survival. This review will outline the various fields of research targeting diagnosis, as well as the modalities used either as primary or as adjunctive treatment of this frequently lethal disease.

EPIDEMIOLOGY
The most common agents of mucormycosis are Rhizopus spp., Mucor spp, and Lichtheimia (formerly Absidia and Mycocladus) spp. Genera of other Mucorales, such as Rhizomucor, Saksenaea, Cunninghamella, and Apophysomyces, are less common.[5] Etiology of mucormycosis varies considerably in different countries. For example, Rhizopus spp. (34%), Mucor spp. (19%), and Lichtheimia spp. (19%) were most commonly identified in patients with mucormycosis in Europe.[6] In India, although Rhizopus species are the most common cause of the disease, Apophysomyces elegans, A.variabilis and Rhizopus homothallicus are emerging species and uncommon agents such as Mucor irregularis and Thamnostylum lucknowense are also being reported.[7,8] Another new species of Apophysomyces, namely, A. mexicanus, has been reported from

Mexico.[9]

Most cases of mucormycosis result from inhalation of fungal sporangiospores that have been released in the air or from direct inoculation of organisms into disrupted skin or gastrointestinal tract mucosa.Seasonal variations affect the incidence of mucormycosis, with most infections occurring from August to November.[10] In a recent study, presenting the epidemiology of mucormycosis in Australia, trauma patients were more often infected with uncommon, non-*Rhizopus* spp.; the patients infected with *Apophysomyces* spp. or *Saksenaea* spp. were all immunocompetent, had predominantly acquired infection through trauma, and had infection frequently localized to the skin, soft tissues, and bones.[11]

The incidence of mucormycosis has been increasing in recent decades, mainly due to the growth of the number of severely immunocompromised patients.[2,3] Now mucormycosis cases are being reported from all over the world, but differences in the epidemiology seem to exist between developed and developing countries.In developed countries, the disease remains uncommon and is mostly seen in patients with hematological malignancies (HM).In contrast, in developing countries, especially in India, mucormycosis is more common and cases occur mainly in patients with uncontrolled diabetes mellitus (DM) or trauma.Accordingly, the prevalence of mucormycosis varies from 0,01 to 0,2 per 100 000 population in Europe and the United States of America,[3,15,16] and is much higher in India (14 per 100 000 population).[7]

The most common clinical presentations of mucormycosis are rhino-orbito-cerebral, pulmonary, cutaneous, and disseminated. The percentages reported in the review by Jeong et al. were 34%, 21%, 20%, and 14%, respectively,[13] while in the European study of the Working Group on Zygomycosis the corresponding numbers were 27%, 30%, 26%, and 15%.[6] In patients with HM, the main clinical form of the disease is pulmonary.[6,17] In India rhino- orbito-cerebral presentation associated with uncontrolled DM was the predominant characteristic, and isolated renal mucormycosis has emerged as a new clinical entity.[7]

Mucormycosis in children was recently analyzed in cases extracted from two global registries.[21] Fungal isolates included *Rhizopus* spp. (39.7%), *Lichtheimia* spp. (17.5%), *Mucor* spp. (12.7%), *Cunninghamella bertholletiae* (6.3%), and unspecified species (23.8%). Underlying conditions were HM (46%), other malignancies (6.3%), HSCT (15.9%), solid organ transplantation, trauma/surgery and DM (4.8% each) and a variety of other diseases (7.9%); in 9.5%, no underlying medical condition was found. Neutropenia was recorded in 46% of patients. The main sites of infection were lungs (19%), skin and soft tissues (19%), paranasal sinus/sino-orbital region (15.8%), and rhino-cerebral region (7.9%). Disseminated infection was present in 38.1%.[21] Mortality, in the same study, was 33.3%. In adults, the reported mortality ranges from 20%.[22,23] to 100%, depending on the underlying risk factors, site of infection and treatment.

DIAGNOSIS

CLINICAL DIAGNOSIS

The prerequisites for the diagnosis of mucormycosis are a high index of suspicion, recognition of host factors, and prompt assessment of clinical manifestations.Diplopia in a patient with diabetes or pleuritic pain in a neutropenic host may be a sign of this infection and should lead to the prompt use of imaging modalities and subsequent acquisition of samples for testing by histology, microbiology, and advanced molecular methods.[5] Nevertheless, there are some features which should lead to a higher index of suspicion for invasive pulmonary mucormycosis.These include a history of prior prophylaxis with voriconazole or the emergence of breakthrough fungal infection in an immunocompromised patient receiving agents active against *Aspergillus* but not Mucorales.[25] Corzo-Leon et al. proposed an algorithm for the diagnosis of rhinocerebral mucormycosis in diabetic patients. The list of signs and symptoms that should be considered to be "red flags" includes a cranial nerve palsy, diplopia, sinus pain, proptosis, periorbital swelling, orbital apex syndrome, and ulcers of the palate.[18] Radiologically, multiple (≥10) nodules, and pleural effusion are reportedly more common in mucormycosis.[25]

Another finding on computerized tomography (CT) scan, which seems to indicate the presence of mucormycosis, is the reverse halo sign (RHS).[26] In a recent study, where sequential thoracic CT scans were performed in leukemic patients with neutropenia, the RHS was observed in 15 of 16 patients (94%) during the first week of the disease, while other radiologic findings, such as multiple nodules, appeared later. The authors concluded that in the particular setting of neutropenic leukemic patients with pulmonary infection, the presence of the RHS on CT was a strong indicator of pulmonary mucormycosis.[26] In another study, the CT scans of 24 patients with lung mucormycosis were compared to those of 96 patients with invasive lung aspergillosis.The RHS was more common in patients with mucormycosis (54%) than in those with aspergillosis (6%, $P < .001$),whereas some airway-invasive features, such as clusters of centrilobular nodules, peribronchial consolidations, and bronchial wall thickening, were more common in patients with aspergillosis.[27] While these findings are not conclusive, they may be used as indicators to start aggressive diagnostic laboratory tests.

Microscopic Examination and Culture

Microscopy (direct and histopathology) and culture of various clinical specimens are the cornerstones of diagnosing mucormycosis.

Direct microscopy of clinical specimens, preferably using optical brighteners such as Blankophor.[30] and Calcofluor.[31] White in clinical specimens allows a rapid presumptive diag- nosis of mucormycosis.[32] Hyphae of Mucorales have a variable width (6 to 25 μm), are nonseptate or pauci-septate.[33] and show an irregular,ribbon-like appearance. Fungal elements may easily be seen on hematoxylin and eosin sections; Periodic acid-Schiff or Grocott-Gomori's methenamine silver staining are used to highlight fungal hyphae and hence to evaluate morphology in more detail.[31] Tissue histopathology is dominated by inflammation which may be neutrophilic or granulomatous;inflammation seems to be absent in a few cases,particularly in immunosuppressed patients.[34] Invasive disease is characterized by prominent infarcts and angioinvasion. In cases where nerve structures are involved a perineural invasion may be present. Neutropenic patients display a more extensive angioinvasion when compared to nonneutropenic patients.[30] Histopathological examination of tissue specimens may not always allow a reliable differentiation between hyphae of *Aspergillus* or morphologically related fungi, and hyphae of Mucorales. However, tissue identification is a very important diagnostic tool, since it distinguishes the presence of the fungus as a pathogen in the specimen from a culture contaminant. All Mucorales grow rapidly (3 to 7 days) on most fungal culture media, such as Sabouraud agar and potato dextrose agar incubated at 25˚C to 30˚C.[35,36]

For some species, a microaerophilic environment improves culture yield.[37] Paradoxically, even when fungal hyphae are seen in histopathologic analysis,fungal cultures are only positive in 50% of cases.[38] Hyphae are friable in nature and hence may be damaged during tissue manipulation (avoidance of excessive tissue homogenization is recommended).[39]

TREATMENT

Successful management of mucormycosis is based on a multimodal approach, including reversal or discontinuation of underlying predisposing factors (if possible), early administration of active antifungal agents at the optimal dose, complete removal of all infected tissues and the use of various adjunctive therapies.[61,63] Rapid correction of metabolic abnormalities is mandatory in patients with uncontrolled diabetes and suspected of mucormycosis.Experimental evidence suggests that the use of sodium bi- carbonate (with insulin) to reverse ketoacidosis, regardless of whether acidosis is mild or severe might be associated with better outcome with the disease due to reversal of the ability of Mucorales to invade host tissues.[64] Corticosteroids and other immunosuppressive drugs should be tapered quickly and to the lowest possible dose. Early diagnosis is crucial in order to promptly initiate therapeutic interventions necessary for preventing progressive tissue invasion and its devastating sequelae, minimizing the effect of disfiguring corrective surgery, and improving outcome and survival.[38,65] In this regard, Chamilos et al. showed that delaying effective amphotericin B-based therapy in patients with hematological malignancies for >5 days resulted in an approximately twofold increase in 12-week mortality (82.9% compared to 48.6% for those who started treatment immediately).[65]

Mucoraceous fungi are resistant to most antifungals*in vitro*, including voriconazole. Amphotericin B is the most active drug, except for some *Cunninghamella* and *Apophysomyces* isolates.[66,69] Posaconazole and isavucona- zole are also active,[70] while itraconazole and terbinafine show some activity against certain strains. There seems to be some correlation between the degree of susceptibility of Mucorales isolates to amphotericin B and outcomes.

In a small study by Lamoth *et al*. MIC $\leq$0.5 μg/ml was significantly associated with better 6-week outcome.[71] A similar correlation was reported in mice, where the efficacy of posaconazole was higher in animals infected with strains of *Rhizopus oryzae* that had lower MICs.[72] There are still not enough data to make a strong recommendation, but the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) / European Confederation of Medical Mycology (ECMM) guidelines recommend susceptibility testing to guide treatment of mucormycosis and to establish epidemiological knowledge.[62]

Mucorales have many common characteristics with other moulds, including portals of entry (airways as well as disrupted mucosal and skin barriers), innate host defenses (polymorphonuclear neutrophil and mononuclear phagocytes, specific ligands in fungal spores such as pathogen-associated molecular patterns, and immune cells such as Toll-like receptors) as well as histopathological and clinical features.[73]

In addition, mucormycosis is characterized by extensive angioinvasion that leads to vessel thrombosis and tissue necrosis.[76,77] Angioinvasion results in hematogenous dissemination of the organism, whereas necrosis of the affected tissues prevents penetration of immune cells and antifungal agents to the infection focus.[75] Certain Mucorales, such as *R. oryzae*, have reduced susceptibility to innate host defense as compared to other fungi, such as *Aspergillus* or *Candida*, making them more difficult to treat.[77,78] and, therefore associated with increased mortality.[2,14]

The 2016 recommendations from the European Conference on Infections in Leukemia (ECIL-6), as well as the ESCMID/ECMM guidelines, advocate the use of a lipid formulation of amphotericin B as first-line therapy for mucormycosis.[61,62] The suggested dose for liposomal amphotericin B is 5 mg/kg/day and as high as 10 mg/kg/day for infection of the central nervous system. In the Ambizygo study, performed by the French Mycosis Study Group, patients received 10 mg/kg/day of liposomal amphotericin B for the first month of

treatment, in combination with surgery, where appropriate. The overall response rate was 36% at week 4 and 45% at week 12. Renal function impairment as shown by doubling of serum creatinine level was noted in 40% of patients (transiently increased in 63%).[79] The study was prospective, but uncontrolled, so its results should serve as a basis for further trials.

The optimal doses for antifungal agents are still an issue of controversy. This is true for triazoles, such as posaconazole and isavuconazole. ECIL-6 recommends the use of posaconazole as salvage or maintenance therapy, while the ESCMID/ECMM guidelines propose its use as first line treatment (moderate recommendation) at a dose of 200 mg q6h of the oral suspension. The advent of the intravenous and tablet forms of posaconazole has led to en- hanced bioavailability and increased drug exposure.[80] This may strengthen the position of this triazole in the anti- fungal armamentarium especially against difficult-to-treat mucormycosis.

Isavuconazole is a recently developed triazole, with a wide spectrum of antifungal activity including Mucorales In a multicenter, open-label trial (VITAL trial) 21 patients with mucormycosis received isavuconazole 200 mg once a day (quaque die [qd]) (after six doses of 200 mg q8h) as primary treatment and were matched with contemporane ous controls from a registry of rare fungal diseases, who had received conventional or lipid amphotericin B at a median dose 70 or 325 − 250 mg qd, respectively as primary treatment.[82] Outcomes in the two groups were similar, and isavuconazole was thus deemed to be an alternative to amphotericin B, as first-line treatment of mucormycosis. Although the results are encouraging, the study has some limitations, that is, small size and external control matching, which should be taken into account.

Another option for salvage treatment, proposed by ECIL-6 is the combination of lipid amphotericin B and caspofungin or posaconazole. There are no data to support the use of two antifungals as first line treatment.

Preclinical data showed increased survival in patients receiving deferasirox, an iron-chelator, in combination with a polyene.[86] However, in a prospective, randomized, clinical study (DEFEAT) performed in patients with hematologic malignancies, the group of patients receiving deferasirox had a higher mortality.[87] The study had several limitations, but both ECIL-6 and ESCMID/ECMM have recommended against the use of deferasirox in such patients. However, deferasirox beneficial role as an adjunctive therapy in patients with diabetes has been shown in several case reports.

The duration of treatment with active antifungal agents has not been determined. Active agents that have oral for- mulations such as posaconazole and isavuconazole are pre- ferred because they can be administered for several months, if needed.

Surgery when needed and possible must be very aggressive. Not only necrotic tissues but also surrounding infected healthy-looking tissues should be removed, as the speed of the extension of the infection by the Mucorales hyphae is enormous. Surgery is particularly useful in rhino-orbito- cerebral infection and in soft tissue infection. In cases of a single localized pulmonary lesion, it may be helpful. It is obviously impossible in cases of disseminated mucormycosis or when infection of difficult-to-reach organs (i.e., certain parts of brain or lung parenchyma close to great vessels) exists.

Other adjunctive therapies are the use of hyperbaric oxygen in an attempt to make a more-oxygen enriched cell environment and administration of cytokines at the same time with the antifungal therapy.

Mucormycosis, although relatively rare, poses an important burden on immunocompromised patients, due to its persistently high mortality. The development of newer, more effective, immunosuppressive medications has been associated with an increase of its incidence. Diabetics are also susceptible to this potentially lethal disease, especially in developing countries. There are several studies on its pathogenesis, but there are still many questions to be an- swered. The diagnosis and treatment of mucormycosis remain a challenge.The clinical presentation is nonspecific, and, when it becomes apparent that the patient most probably has mucormycosis, it is often too late to administer effective treatment. Early diagnosis is thus crucial and is the main target of current research.Direct examination, culture and histopathology are the cornerstones of diagnosing mucormycosis, but they are time consuming and lack sensitivity. Newer molecular diagnostic techniques, such as *in situ* hybridization and PCR, offer an alternative which may lead to earlier diagnosis and prompt initiation of treatment. The management of mucormycosis is multimodal, including reversal of underlying risk factors, administration of antifungal agents, surgical intervention and various adjunctive therapies. Timely and adequately dosed antifungal therapy is necessary. Amphotericin B and posaconazole are the most often used medications.

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