Biology, systematics and clinical manifestations of Zygomycota infections

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Abstract

Fungi cause opportunistic, nosocomial, and community-acquired infections. Among fungal infections (mycoses) zygomycoses are exceptionally severe with mortality rate exceeding 50%. Immunocompromised hosts, transplant recipients, diabetic patients with uncontrolled keto-acidosis, high iron serum levels are at risk. Zygomycota are capable of infecting hosts immune to other filamentous fungi. The infection follows often a progressive pattern, with angioinvasion and metastases. Moreover, current antifungal therapy has often an unfavorable outcome.

Zygomycota are resistant to some of the routinely used antifungals among them azoles (except posaconazole) and echinocandins. The typical treatment consists of surgical debridement of the infected tissues accompanied with amphotericin B administration. The latter has strong nephrotoxic side effects which make it not suitable for prophylaxis. Delayed administration of amphotericin and excision of mycelium containing tissues worsens survival prognoses. More than 30 species of Zygomycota are involved in human infections, among them Mucorales are the most abundant. Prognosis and treatment suggestions differ for each species what makes fast and reliable diagnosis essential. Serum sample PCR based identification often gives false negative results, culture based identification is time consuming and not always feasible. With the dawn of Zygomycota sequencing projects significant advancement is expected as in the case of treatment of Ascomycota infections.

Keywords

zygomycosis, mucormycosis, Mucorales, Zygomycota, nosocomial infection, fungal systematics, entomophtoromycosis, pathogenic fungi, mycosis, diabetes, transplant recipient, hematological malignancy, antifungal therapy,

Introduction

Zygomycota is an artificial grouping of distantly related basal fungi. This term is broadly used for fungi belonging to Mucoromycotina, Entomophtoromucotina, Mortierellomycotina, Zoopagomycotina, and Kickxellomycotina. The common name zygomycosis stands for infections caused by organisms which diverged more than 800 MYA (Krings, Taylor, & Dotzler, 2013), preceding land colonization by plants. In consequence it should be broadly recognized that zygomycoses have a variable prognosis depending on the causal agent and patient's condition. Mycology is undergoing a significant shift towards molecular based taxonomy aiming at a more consistent classification and nomenclature. A robust classification reflecting biological similarities is of great significance for medical purposes. The biochemical and physiological variability among fungal taxa lead to measurable effects among them antifungal resistance (Nikolaos G Almyroudis, Sutton, Fothergill, Rinaldi, & Kusne, 2007; Farina, Marchesi, Passera, Diliberto, & Russello, 2012). Fast and accurate species (or at least genus) recognition is essential for a successful treatment in the case of less common fungi. Yet, in most laboratories such in depth classification is not routinely performed, because these genera cannot be easily differentiated for example histopathologically.

The broadly used and recognized term Zygomycota should be abandoned in the future in favor of proper taxonomic names. Since the clinical manifestations and development of the infections with Mucorales and Entomophthorales are significantly different the name "zygomycosis" should also be abandoned in favour of entomophthoramycosis (entomophtoromycosis), mortierellomycosis and mucoralomycosis (mucormycosis). However here, when describing common features of many taxa, we will apply the term Zygomycota. Emerging Zygomycota infections are becoming a serious threat at transplantation and other wards with immunocompromised patients. This phenomenon become more pronounced after the introduction of serum-based detection, diagnostic imaging and modern antifungals (voriconazole) which enabled efficient treatment of aspergillosis (Pongas, Lewis, Samonis, & Kontoyiannis, 2009). Mucorales are the third most common agent of systemic mycoses after invasive aspergillosis and candidiasis in hematology patients (Kontoyiannis et al., 2010). This constantly growing group of susceptible patients together with improvements in the management of invasive aspergillosis and candidiasis result in increased interest in zygomycoses. In contrast to aspergillosis most cases of zygomycosis are fatal despite the administration of antifungal therapy (Lewis et al., 2012; Pagano et al., 2006; Roden et al., 2005). The number of risk patients is constantly growing with the ubiquity of diabetes and immunosuppressive treatment. Furthermore Zygomycota are able to colonize hosts not susceptible to other opportunistic fungal infections among them diabetic patients and intravenous drug users (Roden et al., 2005). Especially representatives of the genus Basidiobolus (Vikram HR, Smilack JD, Leighton JA, Crowell MD, 2012) and of the family Saksaneaceae (Baradkar, Mathur, Taklikar, Rathi, & Kumar, 2008; Thomas, Shah, Mathews, & Chacko, 2008) have been reported to infect healthy hosts.

Here, we revise current knowledge on nomenclature, pathogenesis and diagnostics of zygomycosis (mainly mucormycosis).

Biology

Mucoromycotina representatives are ubiquitous organisms present all over the world in the soil, infecting plants and other fungi (Hoffmann et al., 2013). Importantly, the same species are found in clinical samples and used in food fermentation. Entomophtoromycotina groups more than 250 entomopathogenic, soil and litter associated

species (Gryganskyi et al., 2013). Mortierellomycotina (Wagner et al., 2013) harbor more than 100 taxa of saprotrophic soil organisms.

The knowledge on pathogenic Zygomycota ecology is very limited and often anecdotal. Understanding the lifestyle of pathogenic species is essential to understand the epidemiology and to formulate recommendations for risk patients. In medical mycology species from order Mucorales are the most common Zygomycota representatives.

Mucorales reproduce both sexualy, by zygospores formed after the fusion of hyphae of different mating types, and asexualy through sporangiospores produced within sporangia. However, in most species zygospores production is more rare and the conditions necessary for their formation and germination remain unknown. In turn, asexual spores are produced massively and they can differ in character (Ainsworth, 2008). Mucorales produce not only dry sporangiospores dispersed by the air but also wet sporangiospores, less prone to aerosolization. Spore size and hydrophilic/hydrophobic character impacts the dispersal of fungi. Human infecting Zygomycetes are relatively hydrophilic what limits the everyday exposition to their spores even if they are ubiquitous in spoiled food and pathogenic strains do not differ from environmental samples (Lu et al., 2013).

Even the most ubiquitous among Zygomycota (*Rhizopus* and *Mucor*) are less common in air samples than benign, plant-associated *Alternaria* and *Cladosporium* (Segvić Klarić & Pepeljnjak, 2006)(Vesper et al., 2005) involved in human allergies. The prevalence of Mucorales spores in dust and spoiled food compared to air samples is consistent with the onset of infections after earthquakes and massive inhalation of spores. Human pathogenic Ascomycota are broadly encountered in air samples whereas Zygomycota are less abundant in air samples (Ejdys, 2007; Gniadek & Macura, 2007; I. El-Herte, 2012). In liquid culture *Mucor circinelloides* produces yeast cells, however the role of yeast-forms in infection is unknown (Khan, Ahmad, Brazda, & Chandy, 2009). Furthermore *M. circinelloides* produces sporangiospores of different sizes depending on the mating type variant (Desjardins et al., 2011). The bigger sporangiospores are able to lyse macrophages what makes them more virulent to a mammalian host.

Until recently it was not clear whether the dimorphic switch between aerobic hyphal growth and multi-budded yeast growth under anaerobic/high CO_2 conditions is related to virulence in Mucorales. Lee and colleagues (Lee, Li, Calo, & Heitman, 2013) showed that a calcineurin inhibitor tacrolimus (FK506) prevents hyphal growth of *Mucor* spp. Furthermore, the cAMP dependent protein kinase A (PKA) is involved in the dimorphic transition alike in other dimorphic fungi (*Candida* species, *Cryptococcus neoformans, Cryptococcus gattii* and *Aspergillus fumigatus*). The yeast-locked mutants were less virulent than wild type strains suggesting that hyphae are more virulent or the dimorphic switch is important for the pathogenicity of this fungus. Finally, the authors showed that calcineurin participates in hyphal polarity development and spore size dimorphism. Calcineurin is a promising drug target against many fungal pathogens with dimorphic growth.

Table 1. A summary of differentiating features between filamentous fungi.

Character	**Ascomycota (eg. *Aspergillus*)**	**Zygomycota (eg. *Mucor*)**
Spores	hydrophobic spores (conidia), ascospores	dry and wet sporangiospores, zygospores

Hyphae	narrow (2-3μm), septate, branching in different angles	broad (6-16μm), coenocytic, orthogonal ramifications
Cell wall composition	more glucan galactomannan present	more chitin galactomannan absent

Mucoralean hyphae are often heavy and have a bigger diameter than in Ascomycota. Hyphae diameter and size determine the efficacy of phagocytosis by macrophages. They differ in cell wall composition as well, they have less glucan and more chitin compared to Ascomycota. This change in biochemistry determines antifungal susceptibility/resistance, because most antifungal drugs target either the cell wall machinery or components. They are galactomannan negative thus serum tests based on this feature can differentiate among Ascomycota and Zygomycota, but only confirming the presence of Ascomycota and providing no information about presence/absence of Zygomycota. Zygomycota are resistant to most of the newer antifungals: voriconazole and echinocandins, which are very efficient against *Candida* and *Aspergillus* mycoses. The three orders of Zygomycota infecting humans have different susceptibility/resistance patterns.

In vitro studies revealed the potential for biofilm formation for *R. oryzae*, *L. corymbifera* and *R. pusillus* but not for *A. elegans* (Singh, Shivaprakash, & Chakrabarti, 2011). The biofilm matrix is formed with glucosamine as the dominant dry component.

Table 2. Summary of differentiating features of entomophtoromycosis and mucormycosis

feature	**mucoromycosis**	**entomophtoromycosis**
progression of the infection	rapidly invasive	local
immune reaction	acute	chronic
host immunity	compromised	competent
localization	systemic	cutaneous, soft tissue
lipase	-	+
keratin degradation	-	+
thermotolerance	+	-

Rhizopus oryzae is claimed to possess extraordinary genome plasticity due to a whole genome duplication which resulted in an elevated number of genes including those involved in host-pathogen interactions (Lewis et al., 2012; Ma et al., 2009). Currently more Zygomycota genomes have been sequenced including *Lichtheimia hyalospora*, *Mucor circinelloides*, *Mortierella elongata*, *Rhizopus microsporus* and *Conidiobolus coronatus* what will enable comparative studies and accelerated screening of drug targets. Comparative analyses will facilitate the identification of conserved components of the fungal cell machinery. Based on the sole gene count and genome size comparisons it is clear that *Rhizopus oryzae* is an exception regarding the number of genes in the whole collection of currently sequenced Zygomycetes with the average gene number of 11 thousand genes. Table 3 lists

estimated genome sizes and predicted gene numbers for Zygomycetes present in the JGI sequencing center database.

Table 3. Currently sequenced Zygomycota genomes stored by JGI

Organism and project name	**genome size**	**number of genes**
Coemansia reversa NRRL 1564 v1.0	21,838,014	7,347
Umbelopsis ramanniana AG # v1.0	23,077,072	9,931
Conidiobolus coronatus NRRL28638 v1.0	39,903,661	10,635
Rhizopus microsporus var. *microsporus* v1.0	25,972,395	10,905
Mucor circinelloides CBS277.49 v2.0	36,587,022	11,719
Lichtheimia hyalospora v1.0	33,282,407	12,062
Gonapodya prolifera v1.0	48,794,828	13,902
Mortierella elongata v1.0	49,959,475	14,964
Phycomyces blakesleeanus NRRL1555 v2.0	53,939,167	16,528
Rhizopus oryzae 99-880 from Broad	46,087,117	17,467

Systematics

Fungal systematics have been recently reorganized (Hibbett & Taylor, 2013) what resulted in a temporary chaos in the nomenclature. A revised taxonomic classification has been introduced for several of the human and animal fungal pathogens also from the previous Zygomycota phylum (Gryganskyi et al., 2013; Hoffmann et al., 2013; Wagner et al., 2013). These changes in nomenclature should better reflect biological differences among taxa. A reliable source of valid nomenclature are: Index Fungorum and MycoBank. Zygomycota themselves are currently not a valid taxon, instead more subphyla and orders are proposed.

Only three of 13 orders of known Zygomycetes, namely the Mucorales, the Mortierellales and the Entomophtorales, harbour animal and human pathogens (Voigt, Vaas, Stielow, & de Hoog, 2013). However *Mortierella wolfii* is a cattle pathogen, the documentation of human infections is scarce and no cases were described since 2000. In consequence, we will focus only on Mucorales and Entomophtorales infections, called mucormycosis and entomophthoromycosis respectively). Supplementary Table S1 lists Mucoromycotina, Mortierellomycotina and Entomophoromycotina involved in human infections with synonymic names and examples of case reports. Mucoromycotina group most of the human pathogenic Zygomycota.

Some taxa have been recently revised, deemed species complex and in consequence divided into more taxa. Among them *Apophysomyces* sp. accounts now for four taxa with differences at spore shape and ITS sequence levels (E Alvarez et al., 2010). Due to the introduction of separated taxa in 2010 most literature refers to *A. elegans*. However, future work should refer to the newest nomenclature. A similar pattern involves *Saksenaea vasiformis*, which is now considered a species complex and in consequence was split into more taxa characterized by spore

shapes (E Alvarez et al., 2010). Among the most characteristic pathogenic Mucorales *Rhizomucor variabilis* was moved to the Mucor genus based on molecular characteristic and renamed *Mucor irregularis* (Eduardo Alvarez et al., 2011). Other taxa suspected to be species complex were confirmed as true species. *Rhizopus microsporus* was revised as a single species and its varieties might be just environmental variants. Importantly, foodborne, environmental and clinical strains are indistinguishable (Dolatabadi, Walther, Gerrits van den Ende, & Hoog, 2013). This observation supports the opportunistic character of mucormycoses

Host - fungus interaction

Key factors involved in fungal infections seem to be shared among distantly related fungi pathogenic against different hosts (Gauthier & Keller, 2013; Lee et al., 2013).

Respiratory tract is the main entrance for fungi, among them for Zygomycetes. Inhaled aerosolized spores are removed by the ciliated epithelium cells moves. If some manage to overcome this barrier the alveolar macrophages phagocyte and destroy most spores. Healthy gastrointestinal tract and skin are good barriers to Zygomycetes. It is not surprising that most of skin infections are a consequence of direct inoculation due to severe trauma. Gastrointestinal infections are most common in neonates, which had not developed proper immune mechanisms.

The description of host pathogen interactions at a molecular level shows differences in the mechanisms of aspergillosis and mucoromycosis including host immune response and susceptibility to macrophage induced hyphal damage (Roilides, Kontoyiannis, & Walsh, 2012). Furthermore biological features of the causing agent determine the location and progress of the infection, leading to a more localized infection in entomophtoromycosis and a progressive and potentially disseminated infection in mucormycosis. Entomophtorales specialized in cutin degradation exert keratinolytic enzymes, in consequence entomophtoromycoses are usually superficial and gastrointestinal infections. In contrast with Entomophtorales human infecting Mucorales are usually thermo tolerant what makes them able to invade internal body parts and form a chronic infection.

The interplay between pathogens and hosts varies among fungal agents and host condition. However, some general trends can be drawn. Most of the mechanisms are known from animal data, the latter should be considered with caution as the inflammatory reaction is different in rodents and apes. The role of immune system in preventing and eliminating fungal infections involves an inflammatory reaction. Inflammation triggered by Zygomycota is usually less visible than in aspergillosis. Ascomycota are recognized by both TRL2 and TRL4 receptors whereas Zygomycota are recognized solely by TLR2 receptors (Roilides et al., 2012). There is another major difference compared to aspergillosis regarding the involvement of T lymphocytes. This is because, innate immunity is indispensable to fight a Zygomycota infection and the acquired immunity involvement is not that pronounced. Hovewer, Mucorales specific T cells and NK cells can be found both in patients and in healthy individuals. These findings pave the way for potential diagnostic tests and adoptive immunotherapy (Ibrahim & Kontoyiannis, 2013).

One of the most characteristic features of zygomycoses is their occurrence in diabetic patients. Some aspects of the interplay between the fungus and diabetic patients has been revealed at a molecular level. During the infection *R. oryzae* binds to GRP78 receptors (Ibrahim, Spellberg, Walsh, & Kontoyiannis, 2012; Liu et al., 2010). The expression of GRP78 receptor coding genes is affected by acidosis, iron and glucose levels which are observed in diabetic patients (high blood sugar levels and available iron). In such a scenario *R. oryzae* become more resistant to neutrophils, which are susceptible to low pH conditions. One taxon may produce hyphae and spores of variable size. Phagocyte-induced damage is related to target mass, bigger hyphae are harder to phagocyte. Schmidt and colleagues (Schmidt et al., 2013) demonstrated that both unstimulated and IL-2 prestimulated human NK cells

damage *Rhizopus oryzae* hyphae, but do not affect resting conidia. They concluded that the damage of the fungus is mediated, at least in part, by perforin. *R. oryzae* hyphae decrease the secretion of immunoregulatory molecules by NK cells, such as IFN-γ and RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted), indicating an immunosuppressive effect of the fungus.

A study on the activity of human polymorphonuclear leukocytes (PMNLs) against *R. oryzae*, *R. microsporus* and *L. corymbifera* revealed that interferon (IFN)- gamma and granulocyte-macrophage colony-stimulating factor (GM-CSF) augment the hyphal damage of all 3 zygomycetes. Additionally, this effect was more pronounced against *L. corimbifera* than against both *Rhizopus* species (Gil-Lamaignere et al., 2005).

Genus specific patterns could be drawn from pulled data from multiple infections. It is currently known that Cunninghammella is more aggressive than *Lichtheimia* in diabetes patients (Roilides et al., 2012). It induces low TNFa levels and is resistant to hyphae damage by macrophages. In rare cases can invade an immunocompetent host (Gupta et al., 2011). What suggests some specific properties of *Cunninghammella* compared to other Mucorales. *Cunninghammella* infections have higher mortality rates than other more common infections (Roden et al., 2005).

Epidemiology

Risk factors

There are several risk factors associated with opportunistic fungal infections among them zygomycoses. Diabetic ketoacidosis (B Rammaert, Lanternier, Poirée, Kania, & Lortholary, 2012; Roden et al., 2005), treatment with corticosteroids (Prabhu & Patel, 2004; Zaki, Elkholy, Elkady, & Abdel-Ghany, 2013), deferoxamine in patients on dialysis (Chayakulkeeree, Ghannoum, & Perfect, 2006) and immunosuppressive drugs (Ibrahim et al., 2012) enhance susceptibility to develop zygomycosis. Neutropenia (Ibrahim et al., 2012; Anna Skiada et al., 2013), malnutrition (Sun & Singh, 2011), cytomegalovirus infection (Mysorekar & Rao, n.d.) and extended wounds/burns (Andresen et al., 2005; Ledgard, van Hal, & Greenwood, 2008)) are also predisposing conditions associated with zygomycosis.

According to the main reviews by Roilides (Roilides et al., 2012) and Ibrahim (Ibrahim et al., 2012) neutropenia, diabetes with blood hyperglycemia and low pH, Fe overload, corticosteroid therapy and direct inoculation in result of trauma are the main risk factors. Neutropenic patients are at increased risk, whereas patients with AIDS are not. This is because, T lymphocytes are not key players in fighting against Zygomycota infection. The phagocytes provide antifungal activity via oxidative metabolites and defensins (Ibrahim et al., 2012). Immunosuppression leading to neutropenia encountered in transplant recipients renders them susceptible to zygomycosis. Corticosteroid therapy also influences neutrophiles and their activity against fungi.

It has been found that (Roden et al., 2005) diabetic patients are exceptionally susceptible to sinusitis infections. Diabetic patients have an altered innate immunity with reduced PMNLs chemotaxis, impaired transmission through vascular endothelium and reduced superoxide production (Peleg, Weerarathna, McCarthy, & Davis, 2007; B Rammaert et al., 2012).

Fe overload - iron is indispensable for vital life processes as a such is a limiting factor. Pathogens developed many traits to acquire iron from the host. Zygomycetes are not an exception, here. Deferoxamine therapy is suspected to facilitate Zygomycota infection by making iron available.

Healthy skin provides an efficient barrier against Mucorales, thus skin trauma provides a risk for developing mucormycosis (Ibrahim et al., 2012). This condition is favorable for fast growing opportunistic organisms. Contact with soil, food, water containing fungi can be detrimental for either burned or severely wounded patients.

Table 4. Infection prevalence and its main features (George Petrikkos et al., 2012; Blandine Rammaert et al., 2012; Roden et al., 2005; Spellberg, Edwards, & Ibrahim, 2005).

Underlying disease/condition	**Percent of patients with zygomycosis**	**The most common form of infection**	**Mechanism and predisposing factors**
Hematological malignancy	11-17	pulmonary; dissaminated	neutropenia, voriconazole prophylaxis
HSCT recipients	1,2-5	rhinocerebral; pulmonary	steroid prophylaxis for GvsH diseas, neutropenia, voriconazole prophylaxis
Diabetes mellitus	17 - 36(74)	rhinocerebral, sinus	acidosis - increasing iron level acidosis and hyperglycemia - negatively impact neutrophil chemotaxins and phaocytic activity
Solid organ transplantation	(0.6)7 - 13 23	pulmonary, liver and other transplant organ	transplant contamination
Deferoxamine therapy	1-6	dissaminated, pulmonary	increasing iron level
Burns, trauma in accident	13 - 20	cutaneous, even 40% of infections get disseminated	disruption of skin barrier (spore inoculation)
Malnourished patients	3	gastrointestinal	digestion of the fungi,
(Premature) neonates	3-21	gastrointestinal	

Sources and routes of infection

Mucorales are ubiquitous in environment. They may be present in rotten organic debris (foods, fruits, vegetables, seeds and nuts, plants). Sporangiospores are easily aerosolized and dispersed in air what is may be the source of infection by inhalation of spores. However, occurrence of sporangiospores in indoor and outdoor air is less than conidia of aspergilli and other molds.

There are three mainly route of infection of Zygomycota. The most common is via respiratory system - sinuses and lungs. The wards where are patients with neutropenia and other risk factor of invasive fungal infection should monitor air-conditioning, and there are not allowed plants as potential sources of fungal growth and sporulation.

Published data describing the levels of zygomycete sporangiospores in outdoor and indoor air in relation to seasonal variation is scarce. It was suggested on the base of two articles from Near East and analyses of seasonal presence of sporangiospores in outdoor air that mucormycosis may occur seasonal in the end of summer and autumn when the month are warm and humid (I. El-Herte, 2012).

The second route of infection is via directly inoculation of fungal propagules into human body. It can be iatrogenic or common acquired. Nosocomial infection can be due to contaminated medical devices: ostomy pouching system, adhesive bandage, wooden tongue depressors, subcutaneous insulin infusion pump, peritoneal dialysis, intravascular devices or contamination during medical procedures: dental extraction and local anesthesia, intramuscular injection of corticosteroids, vitamins and anticoagulant, nasal packing, contamination of grafts and during transplantation. Common acquired inoculation of zygomycota fungi may occur as a result of traffic accident, burns when the contaminated soil, water, plant debris or other things has a contact with injured skin and tissues. Infection can also occur by the insect bites. This route of infections are dominant in healthy patients. The less common of Zygomycota infection is oral route (Blandine Rammaert et al., 2012).

Among nosocomial sources of infection there are reported cases of contaminated biomedical devices and building constructions. In a review of 169 cases by Rammaert and colleagues (B Rammaert & Lanternier, 2012) list a variety of sources of infections among them: grafts, ostomy bags, adhesive bandages, water, wooden tongue depressors, dental extraction and local anesthesia, blood glucose self-monitoring equipment, subcutaneous insulin infusion pump, peritoneal dialysis, intravascular devices, intramuscular injection of corticosteroids and vitamins, nasal packing. Neonatalogical, hematological, transplantation and burn units should monitor their conditions to ensure patients well-being.

Special focus should be directed on donor-derived filamentous fungal infections in transplant recipients (Bakr et al., 2008) which have a fatal outcome (Blandine Rammaert et al., 2012). Environmental sources of infection are dominant in healthy patients with challenged skin barrier - after trauma (car accident, burn) among them soil /decaying organic matter (Austin, Finley, Mikkelson, & Tibbs, 2013) and water (Ribeiro et al., 2010) should be considered. Even plant pots in hospitals can be a source of infection for immunosuppressed patients.

Prevalence

In the general population zygomycosis is a very rare disease. Orphanet (the Orpha number i.e. the Orphanet identifier for zygomycosis is 73263) classified it as a rare infectious disease however its prevalence is not known. There are several estimations of zygomycosis/mucormycosis incidence, showing some discrepancies. A more accurate assessment of zygomycosis prevalence is currently not feasible due to incomplete identification and documentation. However, some best practice approaches have been introduced like the Working Group on Zygomycetes by the International Society for Human and Animal Mycology (ISHAM). Rees and colleagues (Rees, Pinner, Hajjeh, Brandt, & Reingold, 1998) estimated the incidence of mucormycosis to be 1.7 cases per million people per year analyzing a population-based study in USA performed in the 90's. A Spanish multicenter, population-based study, based on 6 diagnosed cases of zygomycosis in Spain in 2005 (Torres-Narbona et al., 2007), showed that the incidence of zygomycosis was 0.43/1,000,000 inhabitants per year and 0.62/100,000 hospital admissions. That gives 430-1700 cases per million per year worldwide. However, Brown (Brown et al., 2012) estimated that more than 10000 life-threating cases of mucormycosis occur worldwide per year with 30-90% mortality rate within infected persons.

Postmortem prevalence evaluation shows that mucormycosis is 10–50-fold less frequent than both candidiasis and aspergillosis with a frequency of 1–5 cases per 10 000 autopsies (Hotchi, Okada, & Nasu, 1980; Tietz, Brehmer, Jänisch, & Martin, 1998; Yanagisawa, Friedman, Kundargi, & Smith, 1977). Zygomycosis represents 8.3%–13% of all fungal infections encountered at autopsy in high-risk patients (G Petrikkos & Skiada, 2012).

Males are more likely affected by zygomycosis than women (58-68% of cases - (A Skiada et al., 2011)(Rüping et al., 2010)(Lanternier et al., 2012)(Roden et al., 2005)). This fact can be explained by hematological malignancies (HM) (and most cancers) incidence being generally lower in women than men. The smaller number of cancers in women is a well-documented phenomenon and could be in part the result of a lower exposure to environmental and occupational risk factors in women than men (Cook et al., 2009; Sant et al., 2010). Nwannadi (Omoti, Nwannadi, Obieche, & Olu-Eddo, 2012) reports that HM more often affects male (56%) and adult (>15yo) 83% patients. However, Skiada found that there is no predilection for sex in cutaneus zygomycosis which affected generally healthy persons (A Skiada, Rigopoulos, & Larios, 2012).

In 2011, a large prospective multinational European study based on 230 patients with zygomycosis showed that the most common predisposing conditions were hematological malignancies (44 %), trauma (17 %), DM (17 %), and hematopoietic SCT (9 %) (A Skiada et al., 2011).

Haematological malignancies

Patients with hematological malignancies (HM) either with or without hematopoietic stem cell transplantation (HSCT) constitute the most commonly infected group. They account for up to 44% percent of all cases of zygomycosis (George Petrikkos et al., 2012; Anna Skiada et al., 2013).

The retrospective cohort study was conducted in hematology wards of either tertiary care centers or university hospitals located throughout Italy, between 1999-2003. Five hundred and thirty-eight proven or probable IFI (invasive fungal infections) on 11802 patients with newly diagnosed hematology malignancies (AML, ALL, CML, CLL, NHL, HD and MM) were documented. The overall incidence of IFI was 4.6%. Zygomycetes in this study occurs in 1,2/1000 (0,12%) HM patients (most frequent among AML and ALL patients - 0,3% patients) with 64,3% mortality rate (attributable mortality rate 0,08%). In this study zygomycetes were fourth agents of IFI following (1) *Aspergillus*, (2) *Candida* and (3) *Fusarium*) (Pagano et al., 2006).

In another study conducted in Southern Italy during 18-months, among 589 (475 adults and 114 pediatrics) patients with newly diagnosed HM (AML, NHL, ALL, CLL, MM) twenty-seven episodes of IFIs were documented (Montagna et al., 2012). The overall incidence was 4.6%. Of the 27 documented IFIs, 16 were caused by yeasts (13 in adult patients and 3 in pediatric patients) and 11 by filamentous fungi (all in adult patients). A total of eleven mold infections (40.7%, 10 aspergillosis and 1 zygomycoses) were reported only in adult patients (incidence 2.3%). Occurrence of zygomycosis was slightly higher than observed in Pagano study (Pagano et al., 2006)- 1,6/1000 *vs* 1,2/1000 HM patients.

Neofytos and colleagues (Neofytos et al., 2009) performed a multicenter, prospective, observational study to assess the epidemiological characters and outcomes of invasive fungal infection (IFI) in hematopoetic stem cell transplant (HSCT) recipients in North America. In this study were included 234 adult HSCT recipients with total 250 cases of IFI. The most frequent was invasive aspergillosis (59,2%) followed by invasive candidiasis (24,8%) and zygomycosis (7,2%), and other molds (6,8%) (Alsharif et al., 2009). In 178 autopsies of HSCT recipients (45,1%) infectious etiologies were found to contribute to the cause of death. Fungal infection were found in 93 cases

(23,5%). Among fungal infection by cultivable species the most common was *Aspergillus* sp. (44,6%) followed Candida (33,9%) and Zygomycota (7,1%) and Pneumocystis (7,1%).

This data suggest that zygomycetes infection occurs in 1,7% patients with HSCT recipients (17 cases of zygomycosis per 1000 HSCT recipients)

Diabetes

The another risk factor is diabetes mellitus. Zygomycosis occur mainly in patients with uncontrolled diabetes especially with ketoacidosis. In 2012 WHO estimated the number of diabetic patients to 220 mlns (current estimates from WHO Fact sheet N°312, Updated March 2013: 347 million people worldwide have diabetes (Danaei et al., 2011)). Zygomycoses are associated with DM in 17-36% of cases. If we take into account estimates that worldwide are 10000 patients with zygomycosis that gives us 0,9-1,8 zygomycosis per 100 000 diabetic patients (approximately 0,0009-0,0018%).

The problem of zygomycosis in diabetic patients is associated with poverty and malnutrition for example in India where uncontrolled DM was the most commonly found predisposing factor in 74% (n = 131) of the 178 cases of zygomycosis identified between 2000 and 2004 (Arunaloke Chakrabarti et al., 2006). In contrast to France where DM was associated only with 9% of 531 cases of zygomycosis reported between 1997 and 2006 (Lanternier et al., 2012).

Solid organ transplantation

Zygomycoses have been reported from different organ such as. renal, liver, heart and lung transplantations. Zygomycosis was generally considered to be a rare complication in solid organ transplant recipients (N G Almyroudis et al., 2006) Incidences of zygomycosis in solid organ transplant recipients are estimated from 0,4 to 16% depending on the procedure and geographical area (Lanternier et al., 2012). The most recent multicenter prospective study conducted in USA found that among 16457 transplant patients 12 had Mucorales infection (0,07%), among particular organ recipients Mucorales infection occurred as follow: lung 2/1179 - 0,17%, liver 7/4361 – 0,16%, kidney 3/8494 - 0,03%; pancreas (1174) , heart (1159), small bowel (69) there were no zygomycosis infection (Kontoyiannis et al., 2010).

Although zygomycoses are relatively rare they are characterized by a severe course and high mortality rate. Early diagnosis of the infection is still a challenge and the demand for better diagnosis is growing with the increasing number of reported cases. The latter is possibly caused by a species shift resulting from the common prophylaxis of fungal infection with azoles (fluconazole and voriconazole) at risk wards. Zygomycota are not susceptible to azoles except for posaconazole.

Clinical manifestations

There are five main clinical manifestations of Zygomycosis (G Petrikkos & Skiada, 2012) rhinocerebral, pulmonary, gastrointestinal, cutaneous and disseminated. Spellberg and colleagues listed sixth group of miscellaneous mucormycosis presentation in which, they included brain involvement without sinus infection, endocarditis, pyelonephrititis, bones infection, external otitis etc (Spellberg, Edwards, et al., 2005).

Among patients from group of risk of zygomycosis the most common manifestation is rhino-(orbital)-cerebral (33-49%), followed by cutaneous (10-16%, pulmonary (10-11%), disseminated (6-12%) and gastrointestinal (2-11%). In immunocompetent/otherwise healthy patients the most representative clinical form was: cutaneous/subcutaneous form (42.5% of patients), followed by the rhino-orbito-cerebral (38%) and genitourinary (8.5%), disseminated and pulmonary infections (Mignogna, Fortuna, & Leuci, 2011).

Rhinocerebral mucormycosis

The rhinocerebral mucormycosis is the most common form present in both neutropenic hematologic and diabetic patients. Initial symptoms are nonspecific involving headaches, altered mental status, fever, and eye syndromes lacrimation, irritation, or periorbital anesthesia. Unilateral vision disturbance and further changes involving ptosis, proptosis or loss of extraocular muscle function are signs of the progressing infection towards the retro-orbital region or the central nervous system (CNS). Necrotic black lesions on the hard palate, necrotic turbinates, septum perforation should be carefully inspected. The radiographic image of the infection can be mimicked by bacterial and other fungal infections as well as tumors. Scheckenbach and colleagues (Scheckenbach et al., 2010) reported that initial CT scanning was not informative and most manifestations were not specific, only histopathology provided sufficient data to establish a reliable diagnosis. A proper management of the primary disease is essential for a favorable outcome.

Pulmonary mucormycosis

Pulmonary mucormycosis usually occurs in patients with profound neutropenia, hematologic malignancies, on corticosteroid therapy or diabetes. The symptoms are not specific and involve fever, chest pain and cough. Neutropenia and coagulation dysfunctions make tissue collection inadvisable in some cases. Radiographic or CT scans showing multiple (>10) nodules or pleural effusions can be a sign of fungal infection. Although other mycoses, bacterial infections or malignant invasions should be considered.

Gastrointestinal mucormycosis

Gastrointestinal mucormycosis is a very rare form of infection. It is associated with severe malnutrition and premature birth. The infection is thought to be a consequence of the ingestion of fungi. The symptoms including fever, pain, vomiting, diarrhea, and constipation are non-specific. The infection might lead to ischemic infarction and ulceration. Stomach, terminal ileum, and colon are the most common sites, however any part of the gastrointestinal tract can be colonized. The diagnosis requires direct examination and biopsy, and is often only performed at autopsy.

Cutaneous (skin) mucormycosis or entomophtoromycosis

Cutaneous infection can be either primary or secondary. A primary infection is developed in patients who suffered burn or other trauma. The infection is caused by direct inoculation during an accident or nosocomial. The disease manifestation involves erythema, pus, abscess formation, tissue swelling, necrosis and pain of the infected area. The skin appears red and indurated and the necrosis can be observed as black eschars. The tissue necrosis can progress to gangrenous cellulitis.

Cutaneous infection can be a secondary locus in patients with disseminated infection from respiratory tract. Cutaneous mucormycosis has been reported in diabetic patients.

Disseminated mucormycosis

This form of mucormycosis is the hardest to control and constitutes the greatest threat to the patient. When two or more non-contiguous organs are involved an infection can be considered disseminated. These often include lung and CNS colonization, with lung infection being often the primary infection site. The involvement of lung and skin is a sign of higher risk of CNS invasion as well. Since the disseminated form often involves CNS colonization the disease can by manifested by changes in the mental status. Other internal organs can be secondarily invaded during colonization among others the spleen, liver and even heart leading to pain of the infected organ.

Pediatric cases

Zaoutis and colleagues consider that pediatric cases should be treated as a separate category (Prasad, Vaughan, & Zaoutis, 2012; Roilides, Zaoutis, Katragkou, Benjamin, & Walsh, 2009). There is limited information about zygomycoses in children. Infection in children is different: they tolerate more intensive treatment and GI tract infections are fatal, being the third most common of zycomycoses manifestations in neonates.

Diagnosis of Zygomycota infections

Zygomycosis is still a challenge for diagnostics especially considering that the early diagnosis is the best drug in zygomycoses. The diagnosis relies on clinical findings, risk factor analysis, histopathology and culture samples inspection. The clinical signs vary with the form and stage of the infection. In the rhinocerebral zygmycosis classic clinical features include facial swelling, with ocular involvement. Patients often report facial and eye pain, proptosis and visual disturbances that are signs of the involvement orbital structures (muscles, nerves and vessels). Black necrotic lesions can also occur for example on the hard palate. Pulmonary zygomycosis manifests itself with nonspecific symptoms such as fever, cough and dyspnea; when fungi invade vessels also hemoptysis may occur. Radiographic findings can be vague and often overlap with other mold infections. The presentation of pulmonary zygomycosis often resembles that of an invasive aspergillosis in severely immunocompromised patients for example HSCT recipients. Comparison of the CT imaging features of pulmonary zygomycosis and invasive pulmonary aspergillosis has shown that the presence of multiple nodules (>10) and, to a lesser degree, the presence of pleural effusions, favored the presence of the former diseases. Additionally, micronodules seen on the initial CT scans were more commonly observed in patients with pumonary zygomycosis (Chamilos, Marom, Lewis, Lionakis, & Kontoyiannis, 2005). Gastrointestinal zygomycosis may occur in patients with severe malnutrition. Clinical picture mimics intra-abdominal abscess. Unfortunately the diagnosis is often made at autopsy. Differentiation with other fungal infection is very important because Zygomycota are resistant to voriconazole, a drug successfully used in the treatment of invasive aspergillosis.

The diagnosis can be performed on several levels i) identification of an invasive mold infection ii) distinguishing between Ascomycota and Zygomycota iii) species/genus identification.

Diagnostic imaging

Diagnostic imaging are useful in early diagnosis of rhinocerebral, non-enhanced paranasal sinuses or lungs lesions. This methods allows to preliminary recognition of probably Mucorales infection. Computer tomography (CT) have a higher sensitivity and allows to earlier diagnosis than classical radiograph techniques. CT scans can reveal lesions caused by angioinvasive fungi not visible in conventional radiographs and prior to other clinical manifestations (Walsh, Gamaletsou, McGinnis, Hayden, & Kontoyiannis, 2012). CT gives opportunity to shows mucosal thickening and early bone destruction. Lesions that could be revealed in lung CT scans are nodules, halo signs, reverse halo signs, cavities, wedge-shaped infiltrates, pleural effusions – angioinvasion of molds. These need to be confirmed by biopsy and molecular or culture based methods. The presence of pulmonary infiltrates in neutropenic patients is already a sign of angioinvasion, necrosis, thrombosis, hemorrhage and edema. These can be the first observable signs of angioinvasion and systemic fungal infection. CT imaging of infected organs often shows limited inflammation around hyphae compared to Aspergillus. The observed lesions can change in the course of infection, but are not enough specific to reliably differentiate among causing agents like Aspergillus, Scedosporium, Fusarium, Mucorales and mixed infections.The major disadvantage of using CT is exposition of

patients on high dose of radiation. The radiation exposure associated with CT scans limits their routinely or serial usage.

Another imaging techniques used in diagnostic is MRI. This method is mandatory for assessing extension into cavernous sinus and identifying cerebral involvement. The lesions which can be revealed by MRI are: orbital and intradural expansion, cavernous sinus thrombosis, or thrombosis or aneurysm of internal carotid artery.

The highest sensitivity among imaging methods of diagnostic has positron emission tomography - PET. This technique can detect small infectious foci before the onset of the anatomical abnormalities assessed by conventional radiology tools. PET is not recommended for the evaluation of cerebral involvement and also in diabetic patients control of glycaemia is required.

Preliminary diagnosis based of above described techniques needs to be confirmed by biopsy and molecular or culture based methods.

Direct examination and culture based methods

Direct examination can provide only limited information on the causing agent. But in the absence of the quick diagnostic test, biopsy with histopathological assessment remains the crucial method in diagnosis of zygomycosis. In tissues materials zygomycosis can be recognized by presence of broad, nonseptate hyphae, invading tissue and cause necrosis, vascular invasion and thrombosis. The hyphae of Zygomycota are branched at a right angle, opposite to other fungi such as Aspergillus which hyphae branch at a slight angles. However they can be easily overlooked in tissue samples stained with non-fungal staining. Many infections get a definitive diagnosis only in autopsy.

For diagnosing of mucormycosis different specimens are taken adequate to the location of the ongoing process of infection. For rhinocerebral form the appropriate samples are as followed sinus aspirates, tissue specimens from affected area and also scraping of the nasal mucosa. In case of pulmonary infection it can be obtained deep samples of tissues and bronchoalveolar lavage (BAL) with flexible fiberoptic bronchoscopy. Sputum testing has lower sensitivity especially in non-neutropenic patients. CT-guided biopsy can be also performed in case of negative BAL findings (Lass-Flörl et al., 2007).

The biopsy of the lesion is necessary to establish the diagnosis of cutaneous mucormycosis . The biopsy specimen should be taken from the center of the lesion and include subcutaneous fat, because molds frequently invade blood vessels of the dermis and subcutis, resulting in an ischemic cone at the skin surface.41 Impression smears from the wound edges may also help in the diagnosis (A Skiada et al., 2012).

Culture is useful for identification of etiological agents and antifungal drug susceptibility testing. Mucorales are fast growing molds, so a 24 h incubation on fungal media like Sabouraud Agar and Malt Extract Agar at 25-37 °C usually suffices for colony formation. A positive culture in a patients with no clinical symptoms should be interpreted with caution because of possibility of environmental contamination. Besides classic morphological identification of molds there are some metabolic markers important for species/genus identification such as ability to grow in certain temperature ranges and assimilation of different compounds as a sole carbon sources.

Human pathogenic Mucorales differ in carbon sources utilization patterns (Schwarz, Lortholary, Dromer, & Dannaoui, 2007), what could be used for species identification either directly in culture dependent assays or for the development of metabolite based approaches. There is limited data on species differences in pathology/treatment due to very recent establishment of some of the aforementioned new taxa. Differential carbon source usage was reported for Apophysomyces with negative results for D-galactose and other sugars (Eduardo

Alvarez et al., 2010)assimilation and positive for the members of six other genera of pathogenic Zygomycota, i.e. Cunninghamella, Lichtheimia, Mucor, Rhizopus, Rhizomucor and Syncephalastrum (Schwarz et al., 2007). Such differences might be used to develop identification tests.

Species identification can be confirmed by molecular methods based on sequencing of a standard PCR product obtained with universal fungal ITS primers on DNA extracted from a culture enables reliable order/species level identification of the infecting fungus. A second possibility in laboratories equipped with reference libraries is techniques such as MALDI-TOF from culture samples.

Culture independent methods - Molecular diagnosis

Currently there are no circulating antigen detection tests available. The promising 1,3 beta-D-glucan tests are commonly found to be negative in Mucorales. A double galactomannan and 1,3 beta-D-glucan test can be of help in excluding invasive aspergillosis or a double infection by Aspergillus and Mucorales. PCR based tests of blood samples are not yet available. In consequence biological samples of the infected sites should be collected for histopathology and culture. Invasive diagnostics biopsy of infected tissue in sinus and lung infection should be performed. Blood and cerebrospinal fluid are usually culture negative.

Culture independent molecular techniques are being developed some are complex and laborious (Zhao et al., 2011) other require sophisticated equipment and reference datasets. Zhao and colleagues describe an assay based on a combination of broad-range PCR amplification and reverse line blot hybridization (PCR/RLB) which enabled them to identify fungi from tissue samples in 7h hours (Zhao et al., 2011) with high specificity (no false positives) and false negatives solely for fungi not included in the reference. Additionally their methods works for concentrations of 1.8×10^{-3} ng/µl of genomic DNA.

More specific and sensitive molecular markers, secondary metabolite identification methods are still being developed.

MALDI-TOF diagnostics are currently based on reference datasets and require cultivation however a development of serum based profiles is expected in the closest future.

Treatment

According to the largest meta-analysis performed by Roden and colleagues (Roden et al., 2005) lack of treatment had a fatal outcome with only 3% of survival. A double treatment consisting of antifungal therapy combined with surgical interventions was the most successful leading to the survival of 70% of patients.

Early diagnosis is the key factor for a successful outcome (Roden et al., 2005). This is followed by surgical debridement and antifungal therapy. Necrotic tissue resulting from disease progression may require repeated excision as it prevents antifungal agent penetration (Anna Skiada et al., 2013). Amphotericin B is the main drug used in the treatment of mucoromycosis, entomophtoromycosis and mortierellomycosis. High therapeutic dosage should be achieved as soon as possible. Lipid formulations tend to be better tolerated, limiting the nephrotoxicity (Antoniadou & Dupont, 2005). Currently, no other drug displays comparable activity against all three groups of fungi. The main antifungal drugs belong to five different groups echinocandins, azoles, polyenes, allyamines and pyrimidine analogues. The echinocandins (caspofungin, micafungin and anidulafungin) inhibit the synthesis of 1,3-β-D-glucan, an essential component of the fungal cell wall. Azoles (fluconazole, hexaconazole, isavuconazole, itraconazole, posaconazole, ravuconazole, voriconazole) are lanosterol 14 alpha-demethylase inhibitors. Polyenes bind ergosterol a cell membrane component (Amphotericin B, nystatin, and natamycin). Allylamines inhibit

squalene monooxygenase among them only Terbinafine is used against fungi. The main antifungal pyrimidine analogue and thymidylate synthase inhibitor is Flucytosine.

An evaluation of amphotericin B, ketoconazole, fluconazole, intraconazole, voriconazole, posaconazole, caspofungin and flucytosine activity against most of the human pathogenic Mucorales: *Rhizopus* sp., *R. arrhizus*, *R. microsporus* var. *rhizopodiformis*, *R. microsporus* var. *micorsporus*, *Mucor* sp., *M. circinelloides* group, *Rhizomucor* sp., *Absidia* sp., *Absidia corymbifera*, *Cunninghamella* sp., *Apophysomyces elegans* revealed that posaconazole is the second most promising drug (Lewis et al., 2012). A recent report from the 3rd European Conference on Infections in Leukemia (ECIL 3) provides a detailed guideline regarding diagnosis and treatment and lists yet unanswered questions (Anna Skiada et al., 2013).

Amphotericin B

Amphotericin B deoxycholate was introduced more than 50 years ago and become the gold standard of therapy of invasive mycoses (Antoniadou & Dupont, 2005; Klepser, 2011). It has a broad spectrum activity against most fungal agents and serves as a reference in searches for novel antifungal drugs. The administration of AMB causes severe side effects among them high nephrotoxicity what lead to the development of less nephrotoxic lipid formulations. The latter can be administrated in higher doses than the native drug. Comparative trials are performed between the more common Liposomal amphotericin B (LAmB) and Amphotericin B colloidal dispersion (ABCD) which is less studied.

Posaconazole

Posaconazole is an exception among azoles which display either moderate or no activity against most of the tested strains . Mucorales are in general susceptible to amphotericin B, but display variable response to posaconazole. A recent report from the SEIFEM and FUNGISCOPE (Pagano et al., 2006, 2013) registries evaluated the outcome from combined lipid formulation of amphotericin B (LAmpB) posaconazole (POS) therapy of invasive mucormycosis in 32 patients (Pagano et al., 2013). This evaluation was essential due to limited number of cases in previous clinical reports and inconclusive previous studies which reported synergistic effects *in vitro* and no improvement relative to LAmpB monotherapy in mouse models. Pagano and colleagues conclude that a combined therapy should be considered in severe cases of invasive mucormycosis. Additionally, it is recommended to consider prophylaxis with posaconazole in risk groups. It is a broad spectrum drug protective against Zygomycota and *Fusarium*, *Penicillium*, *Histoplasma*, *Blastomyces*, *Coccidioides*, *Paracoccidioides*, *Sporotrix*, chromoblastomycosis, mycetoma, phaeohyphomycosis, including *Scedosporium apiospermum* and *Exophiala*, *Alternaria*, and *Bipolaris* species (Scheinfeld, 2007). Among others it has been used for successful treatment of *Cunninghamella elegans* infection (Garbino, Myers, Ambrosioni, & Gumy-Pause, 2010). Furthermore it is the most active drug in *A. elegans* treatment, has lower MIC than AmpB (A Chakrabarti et al., 2010). Saksenaeaceae (Apophysomyces and Saksenaea) seem to be most responsive to antifungals posaconazole, itraconazole, and terbinafine (Hospenthal et al., 2011)and not AmpB.

Vorionazole

Previous exposure of *Rhizopus oryzae* to voriconazole enhances virulence in animal models (Lamaris et al., 2009; Lewis, Liao, Wang, Prince, & Kontoyiannis, 2011)) It is discussed whether the co-occurrence of voriconazole application in antifungal prophylaxis in immunocompromised patients with the frequency of zygomycosis has a causal character (Pongas et al., 2009)

Caspofungin

Reports on caspofungin treatment show discrepancies between in vitro and in vivo results. Caspofungin is claimed to facilitate the development of some zygomycoses similarly to voriconazole and is associated with a worse outcome (A Skiada et al., 2011). On the other hand caspofungin shows synergy with AmpB in mice model (Spellberg, Fu, Edwards, & Ibrahim, 2005). There is a repeatedly confirmed paradox of caspofungin efficacy decreasing to control level, when administrated in higher doses in murine models (Lewis, Leventakos, Liao, & Kontoyiannis, 2011).

Terbinafine

Recently, terbinafine has been successfully applied to patients with mucormycosis (Keller, 2012; Pérez, 1999; Revankar, Nailor, & Sobel, 2008). The drug has been primary against dermatophytes but further usage included Candida and Zygomycota. Terbinafine was the most active compound (among amphotericin B, voriconazole, posaconazole, itraconazole, ravuconazole, terbinafine, and caspofungin) in an *in vitro* study of antifungal activity against five strains of *Cunninghamella bertholletiae* and four of *Cunninghamella echinulata* (Pastor et al., 2010). However, it is intriguing why in vivo studies did not include this compound. Nevertheless the compound was successfully applied against *Basidiobolus ranarum* in a combination with itraconazole in a rhino-facial infection (Goyal, Gupta, Das, & Jain, 2010).

Combined antifungal therapy

Spellberg with colleagues (Spellberg, Ibrahim, Chin-Hong, et al., 2012) formulated three recommendations to introduce phase III clinical trials with combined therapy: 1) improved survival in relevant animal models of mucormycosis 2) available retrospective data that are concordant with the preclinical models both for efficacy and safety 3) involvement of already approved therapeutic agents. The consortium proposed three potentially effective therapeutic combinations: lipid polyene – echinocandin, lipid polyene/posaconazol – deferasirox, lipid polyene – posaconazole/isovuconazole. The aforementioned (L)AmpB and posaconazole combination was the most commonly used combined therapy in patients with hematologic diseases reported from the SEIFEM and FUNGISCOPE registries. In the aforementioned studies LAmB+POS was administered when antifungal monotherapy failed to produce a response. These patients were treated with a monotherapy for a median time of 18 days before LAmB+POS combined therapy was introduced. A robust and consistent assessment of drug efficacy and combined therapy influence on patients outcome is limited by such factors as i) limited number of cases ii) lack of regular therapeutic drug monitoring iii) uncontrolled impact of surgical debridement iv) burden of underlying diseases.

The concluding remark from Pagano and colleagues analyses (Pagano et al., 2013) was, that a combined antifungal treatment with LAmB+POS may be considered in patients with very aggressive forms of IM. This is the only drug combination which is consistently recommended by independent authorities. However, other combinations have been described as well.

In vitro studies as well as animal studies suggested a synergistic effect of deferasirox in combination with LAmB (Ibrahim et al., 2005). However, a randomized, double-blinded, placebo-controlled trial showed that patients with mucormycosis treated with deferasirox with LAmB had a higher mortality rate at 90 days than patients treated with LAmB with placebo. Authors underlined that population imbalances in this small Phase II study limit possible generalizations. Spellberg and co-authors concluded although no obvious toxicities were identified deferasirox

cannot be recommended as part of an initial combination regimen for the treatment of mucormycosis (Spellberg, Ibrahim, Roilides, et al., 2012).

From the theoretical point of view echinocandins should be active against Mucorales – *R. oryzae* porduces the target enzyme for echinocandins (Ibrahim et al., 2005). *In vitro* studies shows higher MICs for this group of drugs (Vitale et al., 2012). In a mice model of disseminated mucormycosis a combination of caspofungin with ABLC (Spellberg, Fu, et al., 2005) and LAmB with either micafungin or anidulafungin (Ibrahim, Gebremariam, Fu, Edwards, & Spellberg, 2008) markedly and synergistically improved survival compared to either monotherapy or placebo. Reed and colleagues conducted a retrospective study concluding that either ABLC or LAmB combined with CASP improved survival for patients with rhinocerebral mucormycosis compared with polyene monotherapy (Reed et al., 2008). Micafungin was also successfully combined with LAmpB, however systematic trials are missing (Ogawa et al., 2012). This effect was previously observed in an animal model of disseminated mucormycosis (Ibrahim et al., 2008). This all data "provide strong rationale for a phase III, randomized double-blinded, placebo-controlled study to determine whether lipid polyene plus echinocandins are indeed superior to lipid polyene plus placebo therapy" - (Spellberg, Ibrahim, Roilides, et al., 2012).

Alternative therapy

Iron chelators and hyperbaric oxygen

Both iron chelators (deferasirox and deferiprone) and hyperbaric oxygen have been used in some cases. However, they are not enough well documented to get positive recommendation from ECIL 3(Anna Skiada et al., 2013).

Immunotherapy and immunostimulation

Adoptive immunotherapy with T cells after stimulation with cellular extracts of fungi may form the basis for future clinical trials. A simultaneous stimulation with *Aspergillus fumigatus*, *Candida albicans* and *Rhizopus oryzae* extracts enables generation of multi-specific T cells that recognize a wide variety of taxa (Tramsen et al., 2013). This in vitro and in vivo results may be a hope for allogeneic hematopoietic stem cell transplantation recipients with invasive fungal infection. However, there is no sufficient clinical evidence to formulate recommendations to use this approach in systemic zygomycosis (Antachopoulos, Katragkou, & Roilides, 2012).

The ECIL 3 suggest that growth factors granulocyte colony-stimulating factor (G-CSF), granulocyte- macrophage colony-stimulating factor (GM-CSF), and interferon-γ (IFN-γ) should be used in neutropenic patients with mucormycosis. Their administration in other patients needs to be further studied.

Final remarks

The constantly growing number of patients susceptible to fungal infections emphasizes the critical need for better treatment and diagnostics. These can be developed based on rigorous meta-analyses of well described case reports with proper taxon identification, treatment and outcome description.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

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