Diploma thesis

Impact of ITS-based sequencing on antifungal treatment of patients with suspected invasive fungal infections

by

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Graz, am 27.08.2020 *Sara Günter eh*

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List of abbreviations

ABPA	allergic bronchopulmonary aspergillosis
ALL	acute lymphatic leukaemia
AML	acute myeloid leukaemia
BAL	bronchoalveolar lavage
BDG	β-D-Glucan
CNS	central nervous system
COPD	chronic obstructive lung disease
CSF	cerebrospinal fluid
CVC	central venous catheter
DKA	diabetic ketoacidosis
FFPE	formalin-fixed paraffin-embedded tissue
GM	Galactomannan
GRP78	Glucose Regulated Protein with molekular weight78 kDa
GVHD	graft-vs-host disease
HSCT	haematopoietic stem cell transplantation
IA	invasive aspergillosis
IC	invasive candidiasis
ICU	intensive care unit
IFD	invasive fungal disease
IFI	invasive fungal infection
IPA	invasive pulmonary aspergillosis
ITS	internal transcribed spacer
LSU	large subunit rRNA gene
NGS	next generation sequencing
ODI	optical density index
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
SOT	solid organ transplant
SSU	small subunit rRNA gene

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Zusammenfassung

Hintergrund

Während der letzten Jahrzehnte hat die Morbidität und Mortalität von invasiven Pilzinfektionen stark zugenommen. Besonders betroffen sind vor allem immunsupprimierte Patient*innen auf den Intensivstationen (1). Die Diagnostik von Pilzinfektionen ist eine große Herausforderung, weshalb diese oft erst spät erkannt werden und antifungale Therapie dadurch verzögert eingesetzt wird (2). Die Rolle von neuen molekularbiologischen Methoden wie ITS PCR und Sequenzierung in der Diagnostik von Pilzinfektionen und die Auswirkung dieser Technologien auf die antifungale Therapie ist bisher wenig untersucht.

Methoden

Die klinischen Daten der Patient*innen wurden aus dem Datenmanagementsystem „Medocs“ entnommen. Pilzinfektion wurden anhand der EORTC/MSG Kriterien beurteilt (3). Der Einfluss der ITS PCR und Sequenzierungsergebnisse auf die antifungale Therapie wurde anhand der in Fieberkurven und elektronisch dokumentierten Einträgen untersucht. Weiters wurden die Ergebnisse der ITS PCR mit den Ergebnissen der Pilzkultur und Mikroskopie verglichen.

Resultate

Es wurden 71 Patient*innen mit insgesamt 81 Proben in die Studie eingeschlossen (vier Patient*innen hatten 2 Proben und drei Patient*innen hatten 3 Proben). Proben aus dem Respirationstrakt wurden am häufigsten untersucht (20 Proben waren aus Lungengewebe, 8 Proben aus BAL Flüssigkeit). Insgesamt war die ITS PCR in 52% der Fälle positiv (n=42), ein positives Ergebnis in sowohl Kultur als auch ITS PCR lag in 24% der Fälle vor (n=19). Der gleiche Pilz wurde in 14 dieser 19 übereinstimmenden Fälle gefunden. Die ITS Sequenzierung bestätigte eine bereits eingeleitete antifungale Therapie in 19/71 Patient*innen (27%), führte zu einer Änderung der antifungalen Therapie bei 11/71 Patient*innen (15%) oder unterstützte die Entscheidung keine antifungale Therapie einzuleiten (34/71 Patient*innen; 48%). Bei 7 von 71 Patient*innen lag das ITS Sequenzierungsergebnis post mortem vor.

Konklusion

Das ITS Sequenzierungsergebnis führte bei einem relevanten Anteil der Patient*innen zu einer Änderung der antifungalen Therapie, während bei der Mehrzahl der Patient*innen die antifungale Therapiestrategie bestätigt wurde. Die ITS Sequenzierung ist daher neben anderen diagnostischen Methoden ein wichtiger Baustein in der Pilzdiagnostik und der davon abgeleiteten Pilztherapie.

Abstract

Background

Morbidity and Mortality rates of invasive fungal infections have increased considerably over the past decades, especially in immunocompromised patients in ICUs (1). An early, targeted and systemic antifungal treatment is associated with a successful outcome (2, 4). Unfortunately, fungal infections are often detected with delay using current diagnostic procedures. The effect of modern molecular diagnostic procedures like ITS PCR and sequencing on antifungal treatment strategies is unclear. The aim of this study was to investigate the impact of ITS PCR and sequencing in the diagnostic process and treatment strategies of patients suffering from invasive fungal infections.

Methods

Clinical data of selected patients was extracted from the computerised clinical databases "*Medocs*". The results of ITS PCR and sequencing were compared to outcomes of fungal culture and microscopy. The impact of ITS PCR and sequencing on antifungal treatment strategies was investigated by review of electronical databases and charts.

Results

The impact of ITS-sequencing on antifungal treatment strategies in 71 patients (81 samples) with suspected invasive fungal infections was retrospectively investigated. Samples from the respiratory tract were most frequently analysed (lung tissue (n=20); BAL fluid (n=8)). The investigation showed that ITS PCR results were positive in 52% (n=42) of the samples. Additionally, both ITS PCR and cultural results were positive in 24% (n=19) of the samples with concurrent results in 14 out of 19 cases.
ITS sequencing either confirmed already ongoing antifungal therapy (19/71 patients, 27%), led to change of antifungal therapy (11/71, 15%) or supported the decision to withhold antifungal treatment (34/71, 48%) (in seven of 71 patients ITS-sequencing results were obtained postmortem).

Conclusion

ITS-sequencing results led to a change of antifungal therapy in a relevant proportion of patients, while it confirmed therapeutic strategies in the majority. Therefore, ITS sequencing was a useful adjunct to other fungal diagnostic measures in our cohort.

1 Introduction

1.1 Clinical background of fungal infections

During the last decades, the morbidity and mortality of invasive fungal infections (IFI) has continuously increased. This has been the case especially in immunocompromised patients, the increase in solid organ transplantations (SOT) and stem cell transplantations (SCT) and increased use of immunomodulation agents for inflammatory diseases such as Crohn´s disease, rheumatoid arthritis and malignant diseases (1, 4, 5). The 12-month incidence of an IFI in patients at risk was 3,4% (4). It was estimated that fungal infections kill approximately 1.6 million people and infect nearly 5 million people worldwide per year (6). Less than 0.01% of the 5 million different fungi species can infect humans (7).

The primary predictive factor for a successful outcome is an early initiation of targeted systemic antifungal treatment (4), since a delay in the initiation or the absence of appropriate antifungal treatment is associated with poor outcome (8, 9). However, despite the advances made in diagnostics, early diagnosis of fungal infections remains challenging (5, 10) mainly due to low sensitivity of diagnostic tests compared to e.g. autopsy findings (4). Even today approximately 50% of all cases of invasive aspergillosis (IA) are only detected post-mortem (11). Molecular techniques like the broad-range internal transcribed spacer (ITS) rRNA gene PCR and subsequent sequencing are promising tools for a rapid, accurate diagnosis of fungal infections (12). Therefore, the aim of this study is to investigate the clinical impact of ITS PCR and sequencing on antifungal treatment of patients suffering from fungal infections.

As fungi primarily infect critically ill or immunocompromized patients, fungal infections are currently a great challange in intensive care units (ICUs) (13). In one study 51% of patients in ICUs suffered from infections, of which 19% were caused by fungi. Fungi were the third most common pathogens (14). The longer the patient stayed at the ICU prior to the study, the higher was his risk of suffering from fungal infections (14). In patients with immunosuppression and severe organ dysfunction, invasive

fungal infections (IFI) were a main reason for morbidity and mortality (15), no matter if patients were treated in surgical or medical ICUs (16).

Fungal infections are endogenous and exogenous in origin. The endogenous sources are primarily mucous membranes of the gastrointestinal tract, as fungi colonize the intestines as part of the endogenous flora. Especially during or after antibiotic therapy, fungi can multiply and become invasive (17). Exogenous sources for fungal infections are contaminated infusions, intravenous catheters and other biomedical devices as well as improperly disinfected hands of hospital staff. Mould infections are also endogenous in origin and primarily caused by inhaling fungal spores (17, 18).

Fungi have a high virulence, which is described as rapid intra-host growth. That might be a reason for the high mortality rate of invasive candidiasis in patients with stem cell transplantation (SCT) of 80% (9). Fungi can persist in resilient stages like saprophytes, and they can survive independently without a host, e.g. in the form of spores (19).
Furthermore, many fungi are able to colonize and/or infect a broad spectrum of hosts. Some fungi are known to infect more than 500 host species, which is not known from other pathogens (19).

In order to infect humans, fungi have to meet the following four criteria:

- The fungus has to be able to grow at body temperature.
- The fungus must have the ability to break through surface barriers.
- The fungus must have the ability of lysis or absorption of tissue.
- The fungus must be resistant to endogenous defence mechanisms such as the innate or adaptive immune system or high body temperature (7).

1.2 Classification of fungal disease: “possible, probable, proven”

In 2002 the *European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group* published standard definitions for invasive fungal infections (IFIs) for clinical and epidemiological studies (1). These definitions have been developed to help the responsible researchers form fairly homogeneous patient groups and thereby increase the quality of clinical studies. Additionally, they support the simplification of clinical trials concerning the evaluation of new medication or medical treatments and communication between researchers (3).
The definitions set in 2002 only applied to immunocompromised patients with cancer and recipients of hematopoietic stem cell transplants (15, 3). However, IFI affect other patients as well, for example patients with solid organ transplantation or with primary immunodeficiency. Therefore, in 2008, the definitions have been adjusted by the same group to include a wider range of patients with predispositions for IFIs (3). They also adopted a new term: “invasive fungal disease” (IFD) is now used rather than the old term “invasive fungal infection”. The new term should represent the procedural aspect of the disease and highlight that it is not a static infection. However, in this thesis I will continue to use the term invasive fungal infections, as it is the term used in nearly all papers and documents.
These definitions determine the probability of a patient suffering from an IFI.
It should be noted that infections caused by *Pneumocystis jirovecii* are not included in the definitions. In addition, the terms apply only to studies, not to clinical practice (3). However, in the absence of comparable definitions in clinical practise, the *EORTC/MSG* definitions are also used in daily clinical life.

According to the *EORTC/MSG Consensus Group*, the likelihood of an IFI/IFD is divided into three stages, namely “proven”, “probable” and “possible”.
The categories of “proven” and “probable” were adjusted in 2008 to reflect the progress made in diagnostic techniques. The category of “possible” was modified to only include patients with a high probability of an infection caused by a fungus, even without a mycological evidence. An IFI is “proven” when a fungus is detected in the

affected tissue by microscopy or culture. The category is valid despite host factors or clinical features. This means that every patient could fall into this category, despite the lack of immunodeficiency. The category “probable” consists of three core components: host factors (Table 1), clinical criteria (Table 2) and mycological evidence (Table 3). The category “possible” solely comprises two: host factors and clinical criteria. It is important to re-evaluate patients in this category over time as many factors develop as the disease progresses (3).

Table 1: Host factors for “probable” and “possible” IFI (3):

- Neutropenia (<0.5 x 10^9 neutrophils/L respectively <500 neutrophils/mm^3) for more than 10 days in context of the beginning of the IFI
- Allogenic stem cell transplant recipients or solid organ transplant recipients
- Primary severe immunodeficiency
- Connecting tissue disorder
- Prolonged immunosuppressive therapy with Glucocorticoids (minimum 0.3 mg/kg/d) for at least 3 weeks or
- Other T-Cell immunosuppressants like cyclosporine or TNF-α-blockers during the last 3 months

The term “host factor” cannot be used as a synonym for “risk factor” since host factors are clinical features that simplify identify patients with a predisposition to IFI (3).

Table 2: Clinical criteria for “probable” and “possible” IFI (3):

- IFI of lower respiratory tract:
    1 of the following signs on a CT scan:
        dense, well-circumscribed lesions with or without halo-sign
        air-crescent sign
        cavity

- Tracheobronchial IFI:

  Tracheobronchial ulcer, nodule, pseudomembrane, plaque or eschar demonstrated in bronchoscopy

- IFI of the sinuses:

  Sinusitis demonstrated by radiological imaging and 1 of the following:

  acute, localized pain, could radiate into the orbit

  ulcer on the nose with eschar

  spreading from paranasal sinuses across the bone barriers

- IFI of the central nervous system:

  1 of the following:

  focal lesions on imaging

  meningeal enhancement in MRI or CT

- Disseminated candidiasis:

  1 of the following, occurring at least 2 weeks after an episode of candidemia:

  small, target-like abscesses in liver or spleen

  progressive, retinal exudates on ophthalmologic examination

Table 3: Mycological evidence for probable IFI (3):

I. Direct tests: cytology, direct microscopy, culture

   Mould in sputum, bronchoalveolar lavage (BAL) fluid, bronchial brush or sinus aspirate samples, shown by 1 of the following:

   presence of fungal structures indicating a mould

   culture shows moulds like Aspergillus, Fusarium, …

II. Indirect Tests: antigen detection or detection of cell wall components

   Aspergillosis:

   Galactomannan (GM) antigen detection in plasma, serum, BAL fluid or cerebrospinal fluid (CSF)

   IFD other than cryptococcosis or zygomycosis:

   β-D-glucan (BDG) detected in serum

Invasive pulmonary aspergillosis (IPA) is increasingly recognized in ICUs (20). Based on the EORTC 2008 criteria it is difficult to diagnose IPA in the ICU due to the following reasons: first, a lung biopsy is often not possible due to anticoagulation or mechanical ventilation using positive endexpiratory pressure. Second, IPA may occur in patients without classical host factors, i.e. associated with influenza (20). Frequently, radiological findings of IPA in ICU patients are non-specific (21, 22) in contrast to the precisely defined radiological criteria of the *EORTC* classification (3). Third, galactomannan (GM) in serum is rarely investigated in non- neutropenic patients. This drawbacks mentioned above may lead to a delay in the onset of antifungal therapy in ICU patients suffering from IFIs and therefore increase mortality (22).

1.3 Other definitions for invasive fungal infections

In 2019 researchers of the *Mycosis Study Group-Education and Research Consortium (MSG-ERC) and the European Confederation of Medical Mycology (ECMM)* published definitions of fungal infections (3). These definitions include breakthrough infection and other circumstances missing in the *EORTC* criteria described above and enable clinical studies with properly defined fungal infections. The most important definitions added by *MSG-ERC/ECMM* are “breakthrough IFI, persistent IFI, refractory IFI, relapsed IFI”. “Breakthrough IFI” can be applied to “a/any IFI occurring during exposure to an antifungal drug, including fungi outside the spectrum of activity of an antifungal.” A “breakthrough IFI” can occur at any time during antifungal therapy and it is irrespective of the type of antifungal drug administration, i.e. prophylaxis, empirical, preemptive or targeted treatment. The minimum time of exposure solely depends on the pharmacokinetic and pharmacodynamics of the medication in use, if optimal compliance is assumed. The period in which a “breakthrough IFI” can be diagnosed continues beyond the last dose of the antimycotic and depends on half-life and dose. If the first signs and symptoms of the IFI occur within less than one dosing interval after the discontinuation of the medication, it should be classified as a “breakthrough IFI”.

A “persistent IFI” is an infection that is stable and unchanged since the begin of antifungal therapy and needs further treatment. If left untreated, IFI normally progresses in immunocompromised hosts. A stable infection is, therefore, a first sign of successful treatment. However, it may also show a poor response in patients with increased in immunocompetence during treatment.

A “refractory IFI” is diagnosed if the disease progresses under treatment with worsening or new signs, symptoms and radiological imaging. It is a result of non-response to antifungal treatment.

A “relapsed IFI” is an infection that occurs after antifungal treatment. The infection is caused by the same pathogens and on the same body side, but a disseminated infection is also possible. A relapse requires a first response to antifungal therapy and is thereby different from the other defined infections (23).

1.4 The different kinds of fungi

Fungi are eukaryotes. There are six eukaryotic supergroups. Fungi belong to the supergroup “Opisthokonta”, which not only includes fungi but also animals and their microbial relatives (24).

Supergroup	Representatives: Groups	Representatives: Genera
“Amoebozoa”		
	Lobose amoebae	*Amoeba*
	Slime molds	*Dictyostelium*
	Pelobionts	*Entamoeba*
“Chromalveolata”		
	Ciliates	*Tetrahymena*
	Stramenopiles	*Phytophthora*
	Apicomplexa	*Plasmodium*
“Excavata”		
	Diplomonads	*Giardia*
	Euglenozoa	*Trypanosoma*
	Parabasalids	*Trichomonas*
“Opisthokonta”		
	Animals	*Drosophila*
	Fungi	*Encephalitozoon*
	Choanoflagellates	*Monosiga*
“Plantae”		
	Green algae	*Arabidopsis*
	Red algae	*Porphyra*
	Glaucophytes	*Cyanophora*
“Rhizaria”		
	Cercomonads	*Cercomonas*
	Foraminifera	*Allogromia*
	Euglyphids	*Paulinella*

Figure 1: The Eukaryotic Supergroups (24)

Fungi played a substantial part in evolution. Without them, there would be no life on earth as we know it. Even today fungi are essential for the balance of the ecosystems and the biodiversity (25). They break down organic substances like dead animals or plants and return minerals like carbon, nitrogen and phosphorus back to the environment (26).

Fungi live inside the human body as opportunists. They cannot cause infections in immunocompetent hosts. However, under special circumstances, they can lead to life threatening infections. This is the case, for example, in a weak immune system. Most fungal infections are caused by less than 12 different fungi, although several hundred different species are able to cause infections in humans. Some of the most common

fungi causing infections in humans are Candida spp., *Cryptococcus neoformans*, Aspergillus spp. and other moulds (27).

1.4.1 Candida spp.

Candida spp. can live as commensals on human skin, in the genitourinary tract and sometimes even in the respiratory tract. They are, however, predominantly found in the human intestine. 50% of all people harbour Candida in their endogenous intestinal flora without any illness (28, 29). Only if the conditions in the host change the fungi can lead to infections. These changes include impairment of the immune system or breakdown of the mucosal integrity (27). Candida spp. are responsible for the most fungal infections in hospitalised patients worldwide (30).

In the human body Candida has the ability to grow as both yeasts and as a filamentous form. This plays a role in the pathophysiology of the infection (31). Altogether there are approximately 150 different Candida species, of which 15 species are able to cause fungal infections in humans. However, 90% of these infections are caused by 5 different species: 40-60% of all infections in humans are caused by *Candida albicans* and 20% by *Candida glabrata*. *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei* are less common (Table 4) (7, 29). *Candida glabrata* is not as virulent as *Candida albicans* and is associated with a higher age of patients and solid organ transplantation (SOT). This shift in epidemiology from *Candida albicans* to non-albicans species is important because of high rates of resistance against Fluconazol in non-albicans species, the medication of choice against *Candida albicans* during last decades. Actually echinocandins are firs line agents in the treatment of invasive candidiasis (29).

Table 4: The five most common Candida species - Epidemiology and Characteristics (29):

Species	Geographic Concentration	Age Predilection	Relative Virulence	Characteristic Clinical Associations	Fluconazole Susceptibility
C. Albicans	Global	None	High	Fungal sepsis, Endophthalmitis	+++
C. glabrata	Europe, US, Australia	Older	Intermediate	SOT	+
C. tropicalis	SA, US, Asia	None	High	Immunosuppression	++
C. parapsilosis	Europe, SA, Australasia	Younger	Low	Medical device	+++
C. Krusei	Europe, US	None	Intermediate	Hematological malignancy	-

Depending on the severity of the damage of the immune system all of the species mentioned above can cause life-threatening diseases in humans (7). In some cases, a Candida infection has a prognosis as bad as in severe sepsis or multi-organ failure (MOF), with mortality rates as high as 50% (32–35). Candida most frequently affects severe ill patients in ICUs and patients after abdominal surgery (32). The Candida infection rates in ICUs, especially in burn units, are 10-20 times higher as in non-ICUs. This leads to an average of 13 additional days of hospital stays (29).

1.4.1.1 Diseases caused by Candida

Frequent manifestations are superficial candidiasis such as thrush, chronic mucocutaneous candidiasis and vulvovaginits (Table 5). These infections are common and self-limited, and, in hosts with a functioning immune system, they are easy to treat with basic hygiene measures and local ointment (28). The type of infection depends not only on the host´s immune system but also on the Candida species that cause the infection (Table 6) (28).

However, Candida can also cause invasive diseases such as candidaemia and deep-seated candidiasis (Table 5). A deep seated candidiasis is an infection of deep, under normal circumstances sterile tissues, which is infected either directly or indirectly through haematogenous dissemination (36). It can affect various organs, for

example, the lungs, liver, spleen, meninges or the heart. If it affects intra-abdominal organs, Candida can additionally cause abscesses, peritonitis or cholangitis. Candida endocarditis is rare. Less than 5% of all infectious endocarditis cases are caused by this fungus (32).

Sepsis, in the context of candidaemia, is 40 times less common than in the context of bacteriaemia. Approximately every fourth patient with candidaemia develops a septic shock. The mortality, in this case, is high with around 90% (29, 30).

Table 5: Disease caused by Candida spp. (28)

Haematogenous infections	**Non-haematogenous infections**
Candidaemia	***Superficial infections***
Endophthalmitis	Cutaneous candidiasis
Vascular-access-related infection	Oropharyngeal candidiasis
Septic thrombophlebitis	Vaginitis
Infectious endocarditis	
Arthritis	***Deep-seated infections***
Osteomyelitis	Oesophageal candidiasis
Spondylodiscitis	Cystitis
Meningitis	Peritonitis
Pyelonephritis	Tracheitis/bronchitis
Pulmonary candidiasis	
Hepatosplenic candidiasis	

Table 6: Species related Candida infections (28)

Species	**Common clinical features**
C albicans	Mucocutaneous infections: oropharyngeal, oesophagitis, vaginitis
	Deep-seated infections: pyelonephritis, peritonitis
	Haematogenous infections: candidaemia, meningitis, hepatosplenic
C parapsilosis	Candidaemia, deep infections associated with implanted devices, infections related to contaminated solutions
	Responsible for most candidaemia among neonates
C tropicalis	Candidaemia and systemic candidiasis in immunosuppressed patients
	Candidaemia may be associated with severe myalgia and myositis
C glabrata	Systemic candidiasis, candidaemia, urinary tract infections
C krusei	Candidaemia, endophthalmitis, diarrhoea in newborns

1.4.1.2 Risk factors

There are several risk factors and co-morbidities for invasive candidiasis (IC) (Table 7). Comorbidities are often diabetes mellitus, liver cirrhosis and malnutrition (32)..

Table 7: Risk factors for invasive candidiasis include (adapted from (35))

- Severe illness, especially in patients with a long-term ICU stay
- Abdominal surgery, especially in patients with anastomotic leakage and repeated laparotomies
- Acute necrotising pancreatitis
- Hematologic malignant disease
- Solid organ transplant recipients
- Solid organ tumour and chemotherapy
- Neonates, especially with low birth weight, and preterm infants
- Broad-spectrum antibiotics
- Central venous catheters
- Total parenteral nutrition
- Haemodialysis
- Prolonged use of glucocorticoids
- Candida colonisation

1.4.1.3 Pathophysiology of Candida infection

Candida spp. colonise the skin and intestine mucosa of most people (31). Mostly, the route of infection is an autoinfection of these colonisations (26, 29–31). In order to invade the blood stream or other hard-to-reach tissues, Candida needs an impairment in the gastrointestinal system (29). Experiments with mice demonstrate that three mechanisms that foster this translocation: (I) disruption of the normal gastrointestinal microbiome and overgrowth of pathogens, (II) increased permeability of the intestinal mucosal barriers and (III) immunodeficiency in the host (37). After the

fungus has grown through the mucosal barrier, it can lead to candidaemia and candidiasis of deep tissues (Figure 2) (35).

Candida is a dimorphic fungus, it is able to grow in yeast and filamentous forms in the human body. Both forms have the same basic components of the cell walls, but differ in the surface proteome and the amount of individual pathogen associated molecular patterns (PAMPs). PAMPs activate and modulate the immune response (31). Studies suggest that that Candida is less virulent in its yeast, non-filamentous form, as it reduces its ability to resist phagocytosis. Another mechanism of Candida to withstand the immune system is the production of a biofilm on natural and artificial surfaces. A biofilm is a community of organisms embedded in an extracellular matrix. It helps the fungus to protect it from the immune system and, further, from being destroyed by antifungals. Biofilms adhere well to medical devices like central venous catheters (CVC). Their ubiquitous use in ICUs put patients at risk for invasive candidiasis as CVCs are susceptible environments for Candida colonization and subsequent IC (29).

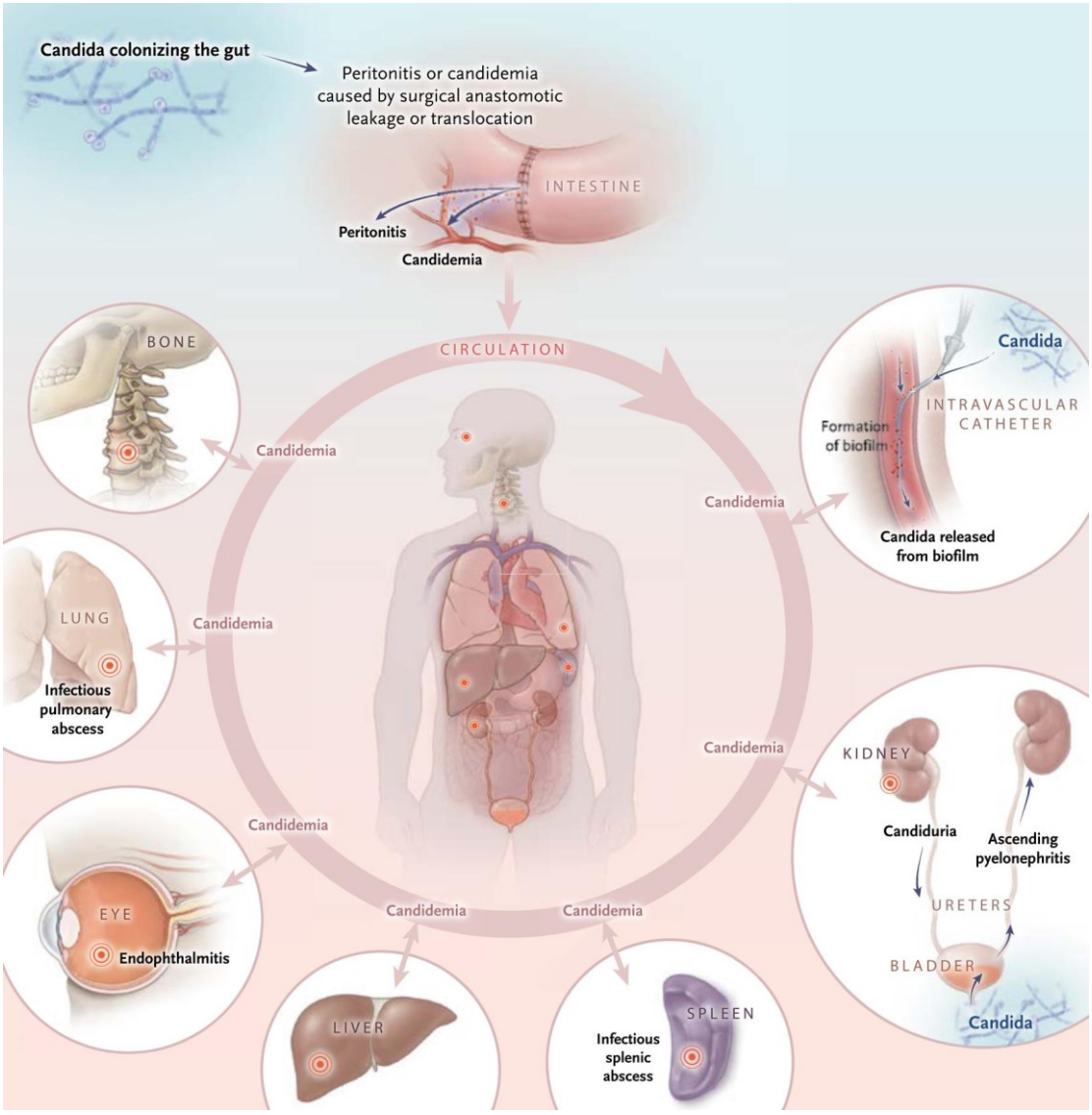

Figure 2: Candida species colonises the gut. They invade through leakage of anastomoses after laparotomy or through translocation, and cause candidaemia or localised infection. (35)

1.4.1.4 Diagnosis

Candida has a creamy or yellowish colour in culture depending on the species. The texture is pasty, smooth, glistening or dry, wrinkled or dull. The microscopic findings depend on the species, as each species shows a typical behaviour. All of them produce blastoconidia, which have round or elongated shape. Most of the species produce pseudohyphae, which are long, branched or curved. Some also produce real hyphae and chlamydospores (28).

Non-cultural diagnostic approaches are the biomarkers β-D-glucan (BDG) and Mannan. The sensitivity and specificity of the biomarkers vary, as e.g. BDG is not specific for Candida. The major benefit of BDG is its high negative predictive value for invasive candidiasis (35).

Gold standard in the diagnosis of Candida spp. is the culture of blood or tissue, but it has its drawbacks, the sensitivity in blood cultures is only 21 – 71%, additional advantages are delayed results or the possibility of inadequate tissue sampling by clinicians. The sample collection from sterile, nonblood sites can be associated with procedural risks. In addition, the growing of the fungus on a culture can be suppressed if the patient has received antifungals (29, 35). A culture is currently the only diagnostic tool that allows routine susceptibility testing.

When diagnosing an invasive Candida infection, there are three clinical situations: (I) candidaemia without deep-seated candidiasis, (II) deep-seated candidiasis without candidaemia, and (III) candidaemia associated with deep-seated candidiasis (36).

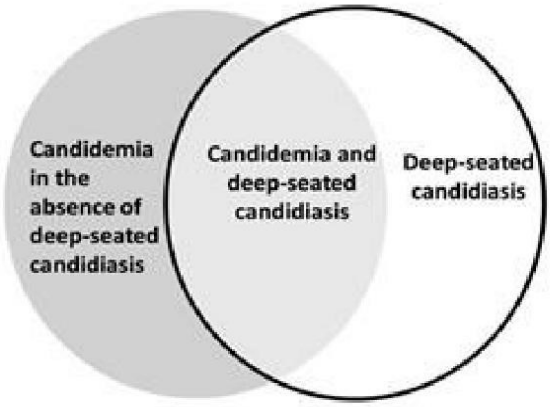

Figure 3: Three entities of invasive candidiasis (36)

Thus, by using blood cultures, the rare diagnosis of deep-seated candidiasis without candidaemia in blood cultures has to be taken into account. For the group of deep-seated candidiasis without candidaemia, diagnostics other than blood cultures are the better choice (36).

In the case of candidaemia, the Candida spp. can be isolated from blood samples. If the patient shows clinical signs of infection, the Candida infection is “proven” according to the *EORTC/MSG* criteria In case of a deep-seated candidiasis, the infection is defined as “proven”, if a culture or histology of normally sterile tissue shows fungal elements (3, 28).

1.4.1.5 Treatment

It is important to start the treatment of a Candida infection as early as possible. Further, adequate source control needs to be initiated in order to decrease mortality (30). The initial targeted therapy should be based on echinocandins (Anidulafungin, Caspofungin, Micafungin) (32). They are favoured over fluconazole because they are fungicide, have a good biofilm penetration, good tolerability and don´t interact with other medicine. It is essential to consider echinocandins´ inability to reach therapeutic levels in eyes, urine and the central nervous system. Hence they do not provide benefit from Candida when the infection spreads in one of those areas. The most frequently used step-down strategy is switching to fluconazole after ten days of echinocandins, if the patient is clinically stable and non-neutropenic with negative blood cultures and fluconazole susceptible Candida species (32).

1.4.2 Aspergillus

Aspergillus spp. are saprophytic and belong to the family of moulds. (38, 39). It is an opportunistic pathogen and has a moderate virulence (40). Due to its ubiquity, it is assumed that every human inhales ten thousands of spores of Aspergillus daily without developing any infection. Only in people with immunodeficiency or severe lung diseases, Aspergillus can cause invasive infections. This is possible since the spores are able to transform into invasive hyphae solely in these people (41).
The genus Aspergillus contains approximately 250 different species. Nearly 40 of them can cause infections in humans. Most infections are caused by *Aspergillus fumigatus*, followed by *A. flavus, A. terreus* and *A. niger* species (42). It is important to identify the species causing the disease because the new antifungals available differ in their spectrum of activity (40).

Worldwide, there are around 200.000 cases of invasive aspergillosis every year with an ascending number. Half of the cases appear in patients with haematologic malignancies such as acute myelogenous leukaemia (AML) and acute lymphatic leukaemia (ALL) and haematopoietic stem cell transplantation (HSCT). In this patient groups the mortality rate is as high as 29% (42).

A. fumigatus has some characteristics that may contribute to its pathogenicity. These include the possibility of growing at 37 °C and a very small spore size of 3-5 µm, which allow the fungus to penetrate deep into the lungs. Additionaly, the spores can withstand many conditions outside and inside the host, because they are surrounded by a hydrophobic protein coat (39).

1.4.2.1 Diseases caused by Aspergillus

The fungus Aspergillus is one of the few organisms which lead to both allergic and life-threatening disease in humans (39). The most important diseases associated with Aspergillus are acute invasive pulmonary aspergillosis (IPA), chronic or allergic bronchopulmonary aspergillosis (ABPA) and aspergilloma, depending on the immune status of the patient. Aspergillus usually infects organs that are in direct contact with the environment, like lungs, sinuses or external auditory canals. Infections can be

locally invasive and destructive and spread especially in immunocompromised patients (40). In healthy individuals, Aspergillus can lead to onychomycosis or tinea cruris. They appear sometimes after changes in either the barriers of skin or mucous membrane or the microflora (43).

IPA is considered the worst disease caused by Aspergillus and affects especially patients with severe immune deficiencies (38), but it is also seen in patients with prolonged neutropenia, solid organ cancer, HIV and organ transplant recipients. During the previous years, an increasing number of patients with prolonged stays at ICUs without neutropenia were affected by the fungus (13). The incidence for invasive aspergillosis (IA) in ICU is around 5.8%, and most of these patients do not have haematologic malignancies but liver failure or COPD (44). The mortality rate in these patients with IA is higher than in patients with neutropenia. Signs and symptoms are nonspecific with fever, cough and purulent sputum. In the early days of the disease, it is indistinguishable from bacterial bronchopneumonia (13). Thoracic pain and haemoptysis can occur as the disease progresses. The disease is able to disseminate into the skin, brain or other organs, particularly in patients with haematologic malignancies (41). It is not uncommon for IA to be associated with respiratory viral infections like adenovirus or influenza (42).

Allergic bronchopulmonary aspergillosis (ABPA) affects patients with asthma (1-2% of asthma patients) or cystic fibrosis (15% of the CF patients) (39). A critical element in the disease is not the mould exposition but the underlying anatomic change in the structure of the lungs. It is a manifestation of hypersensitivity to fungi and particularly to Aspergillus (43). The hyphae grow in the lumen of the bronchi, causing inflammation, bronchiectasis, fibrosis, wheezing and infiltrates (39).

Non-invasive aspergillomas can develop in healed lesions of tuberculosis patients, when they repeatedly come into contact with conidias (38).

1.4.2.2 Risk factors

Patients with severe immune dysfunction are at high risk on developing invasive aspergillosis. These include, for example, patients with haematologic malignancies like leukaemia (38). Other important risk factors are prolonged neutropenia for more than seven days, long-term high-dose corticosteroid therapy, solid organ transplant recipients, especially lung and heart, and stem cell transplant recipients with GVHD as well as patients with hereditary neutrophil dysfunction like chronic granulomatosis (40, 42). Increasingly, invasive aspergillosis is documented in ICU patients not classically considered immunosuppressed (41), these patients are the second largest risk group with 6-57 IA cases in 1000 ICU admissions and mortality rates up to 80% (42). Reasons for the increased susceptibility of these patients to IA are the high use of broad-spectrum antibiotics on ICUs, central venous catheters and mechanic ventilation (44). But not all patients in the ICU have the same risk for developing an IA. Frequent comorbidities include COPD, liver cirrhosis (because of the impaired phagocytosis; however, conflicting results regarding this risk factor are available in the literature (45)), solid organ cancer, diabetes mellitus (because of the impairment of the immune system), alcoholism as well as malnutrition. In patients with septic shock invasive aspergillosis might occur due to the following hypothesis: an initial hyperinflammation is followed by a phase of immune paralysis with deactivation of neutrophils which might allow invasive infections with opportunistic pathogens including Aspergillus (13). In patients with COPD the altered lung structure, reduced mucociliary clearance and mucosal lesions contribute to the high risk of developing an IA. Furthermore, these patients often need broad-spectrum antibiotics, corticosteroids and invasive treatment (46).

Table 8: Risk factors for invasive aspergillosis (44):

- High-risk category:
  - Neutropenia (neutrophil count, <500 neutrophils/mm^3
  - Hematological malignancy
  - Allogenic bone marrow transplantation
- Intermediate-risk category:
  - Prolonged treatment with corticosteroids before admission to the ICU
  - Autologous bone marrow transplantation
  - Chronic obstructive pulmonary disease
  - Liver cirrhosis with a duration of stay in the ICU >7 days
  - Solid-organ cancer
  - HIV infection
  - Lung transplantation
  - Systemic disease requiring immunosuppressive therapy
- Low-risk category:
  - Severe burns
  - Other solid-organ transplant recipients (e.g. heart, kidney or liver transplant recipients)
  - Steroid treatment with a duration of ≤7 days
  - Prolonged stay in the ICU (> 21 days)
  - Malnutrition
  - Post-cardiac surgery status

1.4.2.3 Pathophysiology of Aspergillus infections

Aspergillus spp. produce asexual spores, the conidia, which are released into the air and inhaled by humans. On average one human inhales 200 conidia per day. The conidia of *Aspergillus fumigatus* are 2-3 µm small, which is why they can penetrate deep inside the lungs and adhere to the mucous membrane in the alveoli. In healthy individuals, the conidia get eliminated by either mucociliary clearance or macrophages (38). Phagocytosis is an opsonin-independent and non-oxidative method that is suppressed by corticosteroids through a failure of phagolysosomal fusion and a depressive effect on cytokine production (39). Macrophages are further responsible for the initiation of a proinflammatory response, which recruits neutrophils to the site of infection. If neither of these defence mechanisms stops the conidia from germinate, the hyphae get destroyed by the infiltrating neutrophils.

In IA-susceptible patients, the defences of the lung are compromised leading to fungal colonisation and growth (38). Aspergillus produces a series of virulence factors that enable it to survive in nutrient-low lung tissue and secondary metabolites that alter the functions of the host cells and contribute to the virulence of the fungus (47). Secondary metabolites are mycotoxins, which are gen clusters that have not been fully characterised yet. The one best studied is gliotoxin (47). Gliotoxin acts immunosuppressive by inhibition of the superoxidase release, cell migration, microbicidal activity, cytokine release by leukocytes and cytotoxicity of T-lymphocytes as well as by apoptosis in macrophages (48). In addition, gliotoxin leads to irreversible loss of the blood-brain barrier, which can lead to life-threatening encephalitis (49).

1.4.2.4 Diagnosis

For a positive outcome, a quick diagnosis of Aspergillus down to species level is important because new antifungals differ in their spectrum and mode of action and new emerging species are not as susceptible to different medications (40). However, the diagnosis of Aspergillus is challenging. Clinical signs and symptoms, as well as radiological findings, are not specific, especially in the beginning of the disease, when infection could be treated best. Microbiological diagnosis is limited by the difficulties in obtaining appropriate biopsy material in patients most at risk for invasive aspergillosis (41). Additionally, there is not a single gold standard in the diagnosis of Aspergillus, but a combination of different assays together with clinical and radiological findings (4).

Preanalytical issues that need to be considered are proper selection, collection and transport of specimens to guarantee accurate diagnosis. Specimens of choice are tissues, fluids and aspirates. It is essential to collect specimens before the start of antimycotic treatment.

Microscopy and culture are important tools in the diagnosis of IA. Microscopy has the advantage of being fast. On the downside, the sensitivity depends on the quality of the tissue and the ability of laboratory staff. If fungal components, like hyphae, are

detected in sterile tissue, the infection is considered to be “proven” regardless of the cultural result. Microscopy does not support the final distinction of Aspergillus from other filamentous fungi, but it may give some hints. Aspergillus typically shows dichotomous and septate hyphae, whereas fungi of the order of Mucorales have pauci-septate and 90°C angle branching hyphae (Figure 4). Existing therapy may influence the micromorphology of the fungi, in this case, it should only be noted whether or not the hyphae can be detected.

Culture has the advantage of susceptibility testing. However, it is limited by its duration, since it sometimes takes several days to get results. Additionally, it is unclear if a positive culture shows an invasive infection or just a colonisation. The sensitivity of culture from sputum is 35% and from BAL is 63% (40).

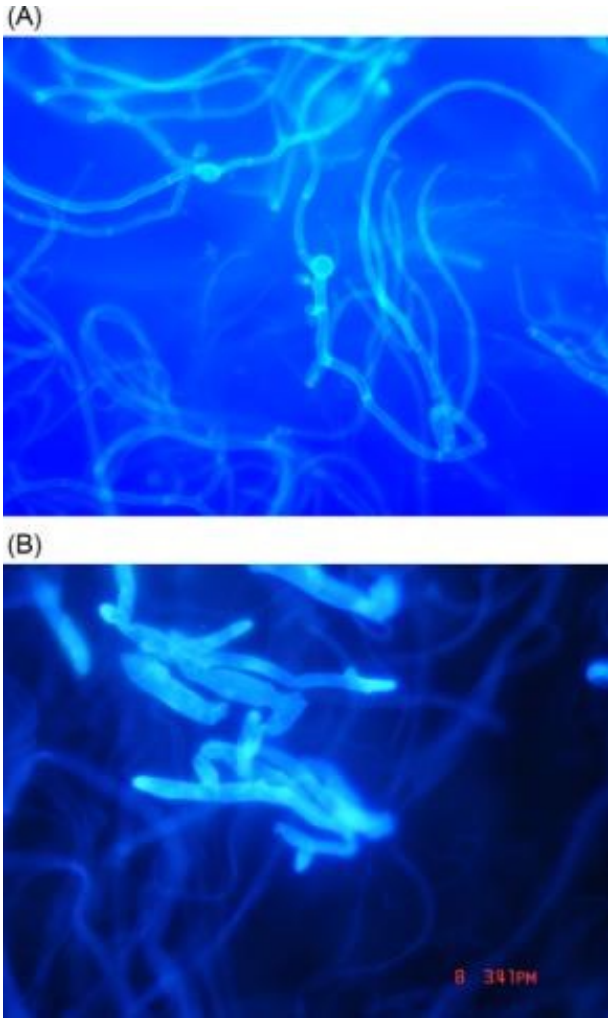

Figure 4: (A) shows hyphae of Aspergillus, (B) shows hyphae of Rhizopus oryzae (Mucorales) (40)

Other diagnostic tools include the antigen assays Galactomannan (GM) and β-D-glucan (BDG). GM is a polysaccharide and a cell wall component of Aspergillus, which proliferates during invasive infections and can be detected in serum and other body fluids. The sandwich ELISA is validated for serum and BAL fluid. It can detect concentrations to a lower limit of 1 ng/ml. GM can be elevated five to eight days before the onset of the first signs and symptoms. Therefore, it is a considerable effective screening test in high-risk patients, but only if they do not receive antifungal prophylaxis, as the positive predictive value is low in this patients. The highest sensitivity of GM is from BAL fluid of patients with neutropenia (40).

BDG is a panfungal marker and not specific for Aspergillus, as it is a cell wall component of species of Aspergillus, Candida, Fusarium, Trichosporon, Saccharomyces, Acremonium and *Pneumocystis jiroveci* and can be released into the body fluids during invasive infections (40). The BDG detection appears to be more sensitive than GM. However, BDG may be falsely elevated in patients treated with immunoglobulins, albumin or other blood products filtered with cellulose membranes (50).

Another useful tool in the diagnosis of Aspergillus is chest imaging. The *ESCMID-ESMM-ERS* guidelines recommendation is a thin-section chest CT with optimised dose (as low as reasonably achievable) or MRI as an alternative (51). Radiographic findings vary significantly depending on host factors. In neutropenic patients, IA presents as pulmonary nodules with surrounding ground-glass infiltrates (halo-sign), showing angioinvasion and haemorrhage into the tissue surrounding the fungal infection, and in later stages of the disease when these nodules cavitate the air-crescent sign may be seen. Despite being typical for IA, these signs are only seen in 10% of the neutropenic patients and rarely in non-neutropenic patients (11).

1.4.2.5 Treatment

The best management of IA is the prevention of the disease, early diagnosis, early initiation of adequate therapy, reduction of immune suppression, if possible, and, if necessary, surgery (4). First-line therapy against Aspergillus spp. is Voriconazole with an improved outcome and decreased toxicity compared with lipid amphotericin B, which is proven to be nephrotoxic (41). The dosage of Voriconazole is 6mg/kg

body weight two times a day as a loading dose followed by 4 mg/kg body weight two times a day (41). Alternatively, Isacuvonazole with its broader activity against moulds can be used for the treatment of IA in patients with haematologic malignancies (5). Its advantages are a long half-life, which allows for a single administration per day (after a two day loading dose) and its water solubility, which makes it less nephrotoxic due to the absence of cyclodextrin, a substance needed in other azoles to increase their water solubility (52). Posaconazole is the recommended antifungal for prophylaxis in high-risk patients, for example patients with prolonged neutropenia after chemotherapy and patients with severe GVHD (5). However, since azoles are frequently used in prophylaxis, an increasing amount of species show resistance against these compounds in some countries like the netherlands (42).

1.4.3 The order of the Mucorales

The Mucorales are an order of filamentous, nonflagellated fungi (53). “Mucormycosis” is the term used to describe infections caused by fungi belonging to the order of Mucorales (41). 75% of all mucormycosis is caused by fungi of the genera Rhizopus, Mucor and Rhizomucor (41). It is the third most invasive fungal pathogen, after Candida and Aspergillus (54). However, the infection still is 10 to 50 times less frequent than candidiasis or aspergillosis (55) with an incidence around 0.5 to 1.2 cases per 1 million people per year (42). But the incidence of mucormycosis is increasing, especially in patients with immunodeficiency, diabetes mellitus and severe trauma, whose wounds are contaminated with Mucorales. Patients diagnosed with mucormycosis have to receive adequate therapy quickly. This therapy needs to consist of antifungals, surgery and, if possible, correction of underlying conditions in order to reduce mortality (13). Mortality rates are as high as 70% in patients with invasive pulmonary mucormycosis (56).

1.4.3.1 Diseases caused by Mucorales

In healthy individuals, Mucorales can cause localized and superficial infections such as tinea cruris or onychomycosis (43). Invasive diseases caused by Mucorales affect almost exclusively immunocompromised patients. The main sites of infection are pulmonary (30%), rhinocerebral (27%), soft tissue (26%) and disseminated mucormycosis (15%) (13, 57). The overall prognosis depends on several factors, including time to diagnosis and to initiation of treatment, the affected body site, the underlying conditions and the immune status of the patient (55).

Pulmonary mucormycosis has roughly the same clinical picture as pulmonary aspergillosis. However, it is more aggressive with high fever, angioinvasion, resulting dyspnoea as well as haemoptysis, chest pain and later cavern formation and tissue necrosis. It additionally affects the pleura, pericardium and mediastinum (41, 54, 58). It mostly affects neutropenic patients with cancer and chemotherapy plus patients after SCT and GVHD (54). Chest images can show infiltration, consolidation,

nodules, caverns, atelectasis, effusions and air crescent sing or reversed halo sign (54).

The initial symptoms of rhinocerebral mucormycosis are similar to the symptoms of sinusitis or periorbital cellulitis with eye or facial pain and facial numbness, conjunctival suffusion, blurry vision and soft tissue swelling. If untreated, the infection can spread into the orbit, where it leads to the loss of extraocular muscle function and proptosis. Initially, the tissue may appear normal on clinical examination, then an erythematous phase with or without edema may occur and at the end, thrombosis of blood vessels and tissue necrosis may result in the development of a black, necrotic eschar. Sometimes, the infection can spread into the mouth and lead to necrotic ulceration of the hard palate or into the central nervous system (CNS) and may be accompanied with a “bloody nasal discharge”. In CT imaging, “the most common finding is a subtle sinus mucosal thickening or a thickening of the extraocular muscles”, and it is also common that the bones appear to be normal (58). Rhinocerebral mucormycosis occurs especially in patients with poorly controlled diabetes, but also in patients with haematologic malignancies, SCT and SOT recipients (54).

Localised cutaneous mucormycosis only affects skin and subcutaneous tissue, but it can spread into deep tissue like muscles, tendons or bones, and even disseminate into non-cutaneous organs. It results from direct inoculation of fungal spores into the skin of patients with open wounds after trauma or burns, dissemination from internal organs into the skin is rare. The clinical manifestation differs; its onset can be slowly or fulminant with gangrene and haematogenous dissemination. Characteristically, it is presented with a necrotic eschar with surrounding erythema and induration (54).

Gastrointestinal mucormycosis is rare. Oftentimes it is only diagnosed post mortem, as the mortality rate is around 85%. It mainly concerns neonates, where it presents as necrotizing enterocolitis, or patients with malnutrition, haematologic malignancies and diabetes. It can develop if pathogens are ingested with food, and it can affect any part of the gastrointestinal tract. Most commonly the stomach, colon and ileum are affected. Usually it presents itself as a perforation with massive bleeding. The liver, pancreas and spleen can also be involved (54).

In the case of disseminated mucormycosis, many organs including the lung, the gastrointestinal tract, the skin and, by dissemination via the bloodstream, the liver,

brain spleen or heart might be involved. Symptoms depend on the organs involved, and, without adequate treatment, it always leads to death (54).

1.4.3.2 Risk factors

Mucormycosis occurs especially in neutropenic patients, for example, with hematologic malignancies or SOT (13). It can also affect immunocompetent patients like diabetics with uncontrolled hyperglycaemia as well as patients with prolonged glucocorticoid therapy and after severe trauma or burns, when fungi settle in the wounds. Other groups at risk for invasive mucormycosis are patients with high serum iron concentrations and intravenous drug users (59). In their study of mucormycosis cases in Europe during 2005 and 2007, *Skiada et al*. demonstrated that the primary site of infection varies according to the predisposing host factor. In patients with diabetes, the mucormycosis mostly affected the rhinocerebral area. In patients with haematologic malignancies, the primary site of infection was mostly pulmonary (60).

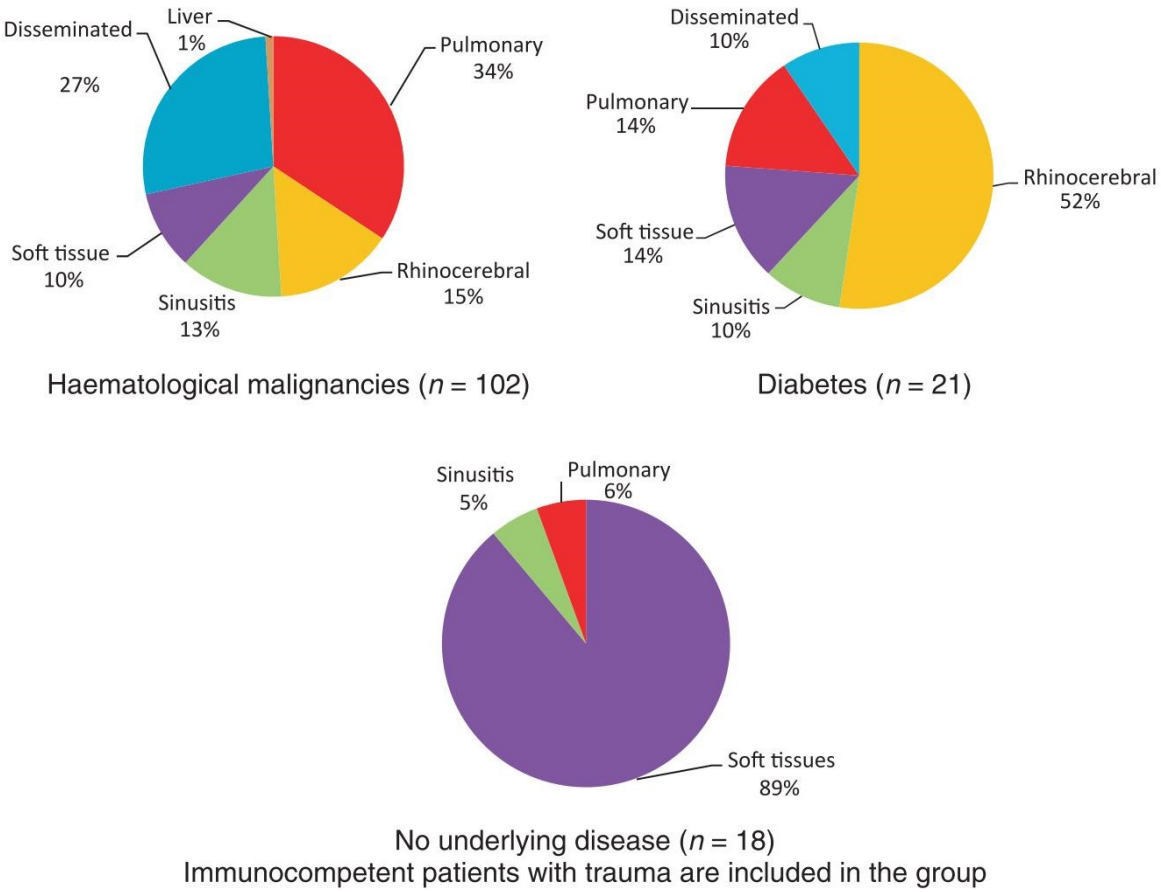

Figure 5: Clinical presentation of mucormycosis in relation to host factors (57)

1.4.3.3 Pathophysiology

There are several ways of Mucorales infection: traumatic inoculation through the skin, ingestion of infested food or inhalation of the sporangiospores in the lungs and sinuses (7, 55). The infection progresses rapidly as the fungus grows quickly through tissue and blood vessels, causing infarction and necrosis (Figure 6) (7). Due to their ability of angioinvasion, the fungus can easily disseminate to other organs. A critical step in the process of angioinvasion is the interaction of the organisms with the endothelial cells and extracellular matrix proteins lining blood vessels. Mucorales can destroy endothelial cells by inducing their own endocytosis into the cells. This endocytosis process is mediated by the binding of the Mucorales to a cell receptor,

the Glucose Regulated Protein 78 (GRP78). GRP78 is a heat shock protein and plays different roles in metabolic processes inside the cell. In stressful situations, it is overexpressed on the host cell surface, e.g. when the endothelial cell is exposed to elevated concentrations of glucose or iron. Consequently, the Mucorales are more likely to invade and destroy the cells. Another reason for patients with high serum iron being at risk on mucormycosis is that iron is essential for the progression of the infection and the survival of the fungus. In healthy hosts, serum iron is bound by iron sequesters, like transferrin, ferritin and lactoferrin. Mucorales can either develop strategies to strip iron from these sequesters, for example siderophore production, or they take up the iron when it becomes more available under certain medical conditions. These are, for instance, hyperglycaemia, acidosis or deferoxamine therapy. Hyperglycaemia leads to the glycosylation of proteins like ferritin or transferrin, which results in decreased iron affinity of the sequesters and an increased release of free iron in the bloodstream. Acidosis causes proton-mediated dissociation of iron from sequesters. Deferoxamine is a bacterial iron-siderophore used to treat patients with iron overload, as it works as an iron chelator in humans. Mucorales use the iron-rich form of deferoxamine, ferrioxamine, as a xeno-siderophore to absorb the iron (61).

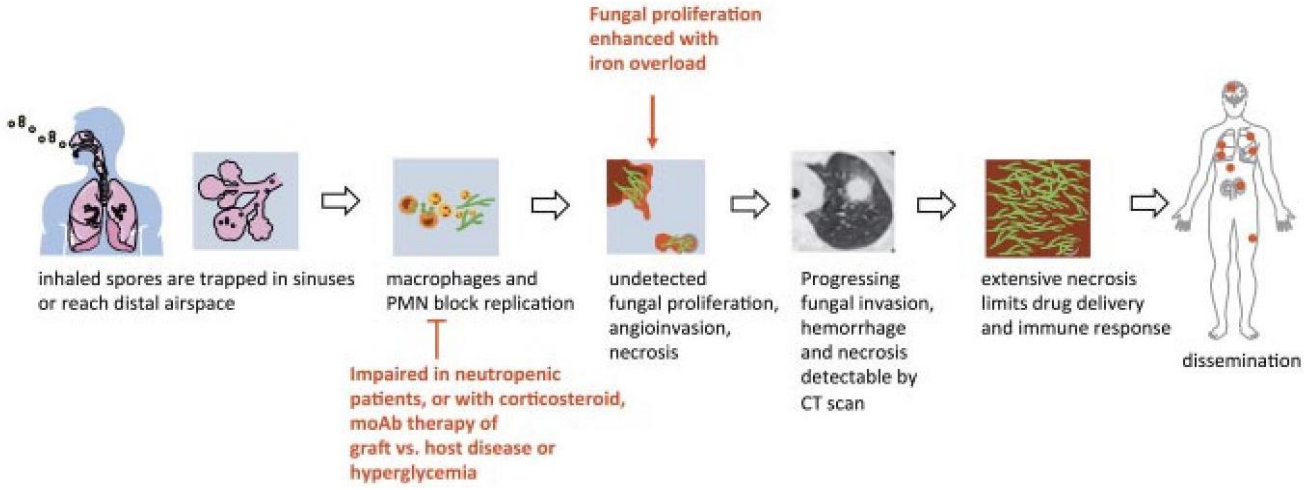

Figure 6: Pathophysiology of invasive mucormycosis (62)

1.4.3.4 Diagnosis

In a 180-day study, *Jeong et al.* found, that a delayed diagnosis of mucormycosis of more than 16 days is associated with increased mortality (59). There are two ways to make a clear diagnosis. Either the fungus can be identified under the microscope or it can be cultured from specimen that has been obtained from the infected area of the body. The fungus has a rather typical appearance under the microscope, the hyphae can be nonseptate or just slightly septate with an inconstant width of 6 to 25 µm and an “irregular, ribbon-like appearance”, and the angle of branching is variable as well. The affected tissue shows infarcts, angioinvasion and perineural invasion. Culture is often negative despite a positive microscopic result, which can be explained by the fragile growth of the organism, as it can easily be destroyed during sample collection and manipulation. The BDG and GM antigen-assays are not suitable for the detection of mucormycosis, and a positive GM or BDG test is a strong evidence against an infection caused by the fungi. However, mixed infections including Aspergillus concomitant to Mucorales sometimes occur (55).

1.4.3.5 Treatment

An early treatment of mucormycosis reduces mortality (figure 7). Antifungal therapy is only one part of the successful management of the infection. It is further important to decrease underlying factors like hyperglycaemia or acidosis and to surgically resect infected tissue, particularly in the rhino-cerebral and skin mucormycosis. This is especially beneficial in case a full resection of invaded tissue cannot be achieved (13). Mucorales are not susceptible against most medication used in Aspergillus infections (63). The first line therapy is lipid amphotericin B, posaconazol can be used as step-down therapy (41). Isavuconazol can be used if intolerances or contraindications prohibit the use of amphotericin B, as it has a broad activity against various moulds (5). Voriconazol is often used as empiric treatment in mould infections due to its activity against Aspergillus, but is has no activity against Mucorales (41).

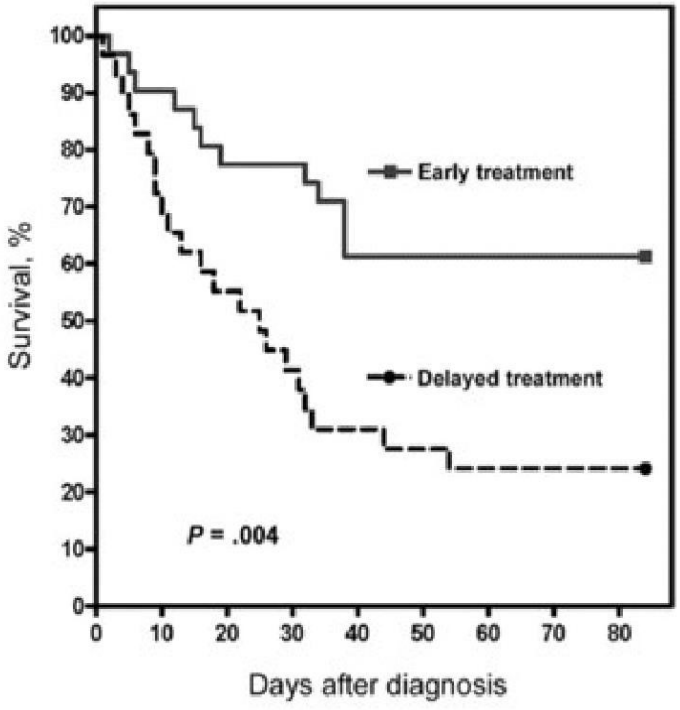

Figure 7: Survival of patients with diagnosed mucormycosis after early (less than 6 days after diagnosis) and delayed (more than 6 days after diagnosis) treatment with amphotericin B (63)

1.5 Routine diagnostic procedures in fungal infections

Opportunistic invasive fungal infections are a major reason for morbidity and mortality in immunocompromised patients, despite the increasing number of antifungals with various spectra of activity (2). However, there are still uncertainties about the optimal way to diagnose these infections. Although there are standards for the diagnosis of IFI, they usually require invasive methods to obtain the required specimens for histopathology and culture, and such methods are often not feasible (1). Even when obtainable, different fungal species can display similar histopathologic features and, therefore, histopathology alone is not a reliable tool for identifying fungi. It should be used in combination with culture. The drawback of cultures is the low sensitivity. Blood cultures have been reported to be negative in approximately 50% of patients with proven candidiasis and in almost every case of aspergillosis or mucormycosis. Additionally, using certain specimen like sputum or BAL, it can be difficult to distinguish between colonisation and true infection (2).

Another important point is that the diagnostic standards are often designed for severely immunocompromised patients. The reliability of these criteria in critically ill ICU patients, although one of the biggest patient group at risk, is questionable (15). Therefore, clinicians often rely on a combination of clinical, radiological and laboratory data. This is less specific, and, therefore, diagnostic uncertainty is one of the main challenges in many fungal infections (1). As a result, the patient is often mistreated for a long time, either with an ineffective antifungal medication or with broad-spectrum antibiotics as long as only bacterial infection is suspected. In addition, using empirical and not targeted antiinfective treatment, the problem of antifungal and antibiotic resistance increases (64). Therefore, additional diagnostic tools, which overcome these shortcomings, are highly needed (12).

1.6 Internal Transcribed Spacer

During the last two decades, culture-independent PCR-based molecular techniques for accurate and rapid diagnosis of fungal pathogens have been introduced (55, 12, 65). The target is mostly the eukaryotic rRNA gene (figure 8), which consists of the 18S small subunit (SSU), 5.8S and 28S large subunit (LSU) rRNA genes transcribed by RNA polymerase 1. Next to the 5.8S rRNA gene are two internal transcribed spacers (ITS), ITS1 and ITS2. As they are not essential in the mature ribosome, they are removed post-transcriptionally (66). Since they are dispensable for ribosomal function, they undergo lower evolutionary pressure which usually results in sequence variability through insertions, deletions and point mutations. This highly conserved ITS genes allow differentiation of even closely related taxa at species level (66, 67). Early PCR techniques addressed the 18S rRNA gene as a molecular target for fungal identification, in analogy to the prokaryotic 16S rRNA gene. However, the 18S rRNA gene is less discriminatory for fungi as the prokaryotic equivalent. It often fails to identify fungi to a lower taxonomic level. Furthermore, ITS sequences seem to have a higher positive PCR amplification rate compared to small subunit (SSU) and large subunit (LSU) sequences, making them a superior molecular target for fungal PCR amplification (66). On this basis, the *Fungal Barcoding Consortium* designated the ITS region as the universal barcode for fungi superior to other molecular markers (68). It has the highest probability of identifying most fungi, because of the most clearly defined barcode gap, meaning interspecific variation exceeds intraspecific variation (68).

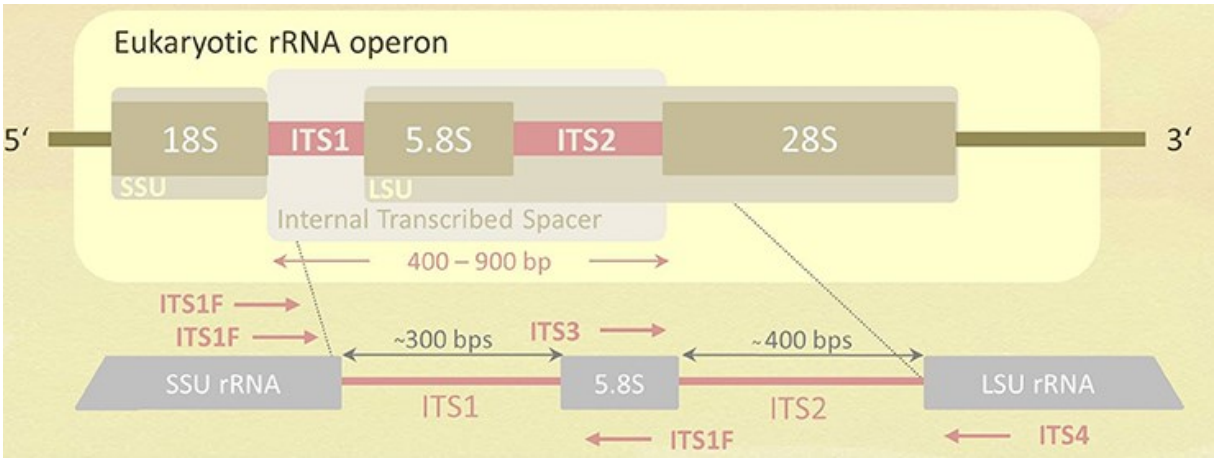

Figure 8: Structure of the ITS rRNA gene (66)

It is possible to choose between different targets within the ITS region. Some favour to target either ITS1 or ITS2, whereas others prefer to amplify the entire ITS region (ITS1 – 5.8S – ITS2). The selection of the primer influences the result. Primers addressing the ITS1 region, like ITS1F, are biased towards amplification of Basidiomycetes, whereas others like ITS4 target the ITS2 region and are biased towards Ascomycetes (figure 8) (69). In formalin-fixed-paraffin-embedded (FFPE) tissue, the ITS2 region shows better results. FFPE samples play an important role in the clinical context, as biopsies obtained during surgery are often fixed in formalin (10%) (66). After amplification, the sequences can be compared to large databased, for example the *UNITE database* (69).

Buitrago et al. compared the results of PCR based methods, histopathological reports and microbiological culture. According to this study, the sensitivity of culture was 56% and of PCR techniques 89.3%. PCR showed positive results in 24 out of 30 patients with negative culture but histopathological proven IFD (65). In other studies, ITS PCR techniques had an analytical sensitivity of 87.7%, analytical specificity of 90.3%, the positive predictive value of 76% and the negative predictive value of 95.5% using culture as the gold standard, with a concordance of 89.6% between culture and PCR results (70). Optimal diagnostic results are achieved through the combination of ITS PCR and biomarkers like BDG or GM (71).

2 Materials and Methods

2.1 Study design

For this single centre, retrospective study, clinical data of patients with ITS PCR and sequencing of tissue or other samples obtained by any kind of invasive or non-invasive procedure were extracted from computerized clinical databases, medical records and handwritten charts. All patients available in the ITS sequencing database of the Institute of Pathology at the Medical University of Graz, were eligible for the study.

78 patients had samples investigated by ITS PCR and sequencing at the Institute of Pathology at the Medical University of Graz between January 2015 and March 2018. The used specimens were mainly obtained from sterile parts of the body, but also from skin, eye and ear-nose-throat areas. All specimens were analysed by either histopathology, microbiology, or both. The results were compared to the results of the ITS PCR and sequencing.

Patients were clinically categorized in “possible”, “probable” or “proven” invasive fungal infection cases based on a recent recommendation of the *European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group* (3). Data was collected about underlying disease, cause of hospital admission, suspected disease before IFI diagnosis, clinical course of disease, microbiological results, additional diagnostics like radiology, serology and pathology, as well as prior anti-infective medication.

Microscopy of samples used for ITS PCR and sequencing was performed by pathologists with material that has also been used for ITS investigation. Fungal cultures were performed in local microbiology laboratories with material obtained in addition to ITS samples during the same sampling procedure. Imaging investigations included CT, MRI, PET CT and leukocyte scintigraphy scans and were analysed by radiologists during clinical routine.

The clinical impact of ITS-sequencing results was assessed by the evaluation of diagnostic and/or therapeutic measures (e.g. new antifungal agent). These measures

were based on the ITS outcomes and documented in the electronic databases (*MEDOCS*) used at the University Hospital of Graz. Additionally, the reason for ITS sequencing, the person or institution ordering ITS sequencing as well as the clinical impact of ITS sequencing results was evaluated.

The study protocol was approved by the local ethics committee, Medical University of Graz (protocol number 31-383 ex 18/19), including a waiver of informed consents as the data evaluation was retrospective in nature.

2.2 The case report form

The case report form included:

- Age at diagnosis
- Gender
- Underlying disease
- Cause of hospital admission
- Diagnosis before ITS PCR and sequencing results
- Diagnosis after ITS PCR and sequencing results
- Material used for ITS PCR and sequencing
- Result of ITS PCR and sequencing
- Presence of an infectious disease specialist consultation
- Who ordered ITS PCR and sequencing
- *EORTC/MSG* criteria
- Histopathology results
- Microbiology results
- Radiological findings
- Anti-infective treatment before ITS PCR and sequencing results
- Therapy modification after ITS PCR and sequencing results
- If there was no modification, what was the reason
- Who initiated the therapy modification
- Outcome
- Cause of death

2.3 ITS PCR and sequencing

A broad-range internal transcribed spacer rRNA gene PCR is performed regularly at the Institute of Pathology at the Medical University of Graz.

2.3.1 DNA Isolation and PCR Amplification

Fungal DNA was extracted with the Maxwell RSC Blood DNA Kit (*Promega, Mannheim, Germany*) according to the manufacturer's instructions with slight modifications. Using the Lysis Buffer stool samples were homogenized on a MagNA Lyser Instrument using MagNA Lyser Green Beads (*Roche Diagnostics GmbH, Mannheim, Germany*). After homogenization samples were treated with 2,5mg/ml Lysozyme (*Roth GmbH, Karlsruhe, Germany*) for 30 min at 37 °C followed by digestion with 1mg/ml Proteinase K for 60min at 56°C. Enzyme activity was inactivated for 10 min at 95°C and 600 µl of lysate was used for the DNA isolation in the Maxwell RSC. DNA concentration was measured by Picogreen fluorescence. The variable ITS region of the fungal 5.8S rRNA gene was amplified with PCR from 20ng DNA using oligonucleotide primers ITS1_fwd: TCCGTAGGTGAACCTGCGG and ITS2_rev: GCTGCGTTCTTCATCGATGC. This ITS rDNA region was chosen since it gives robust taxonomic classification and has been shown to be suitable for community clustering. Fungal 5.8S DNA was amplified with the Mastermix 16s Complete PCR Kit (*Molzym, Bremen, Germany*) according to the manufacturer's instructions using 0.4µM final concentration of primers and 57° annealing temperature for 25 cycles. The first PCR reaction product was subjected to a second round of PCR with primers fusing the ITS primer sequence to the A and P adapters necessary for Ion Torrent sequencing while additionally including a molecular barcode sequence to allow multiplexing of up to 96 samples simultaneously. PCR products were subjected to agarose gel electrophoresis and the band of the expected length (350nt) was excised from the gel and purified using the QiaQick (*Qiagen, Hilden, Germany*) gel extraction system. DNA concentration of the final PCR product was measured by Picogreen fluorescence.

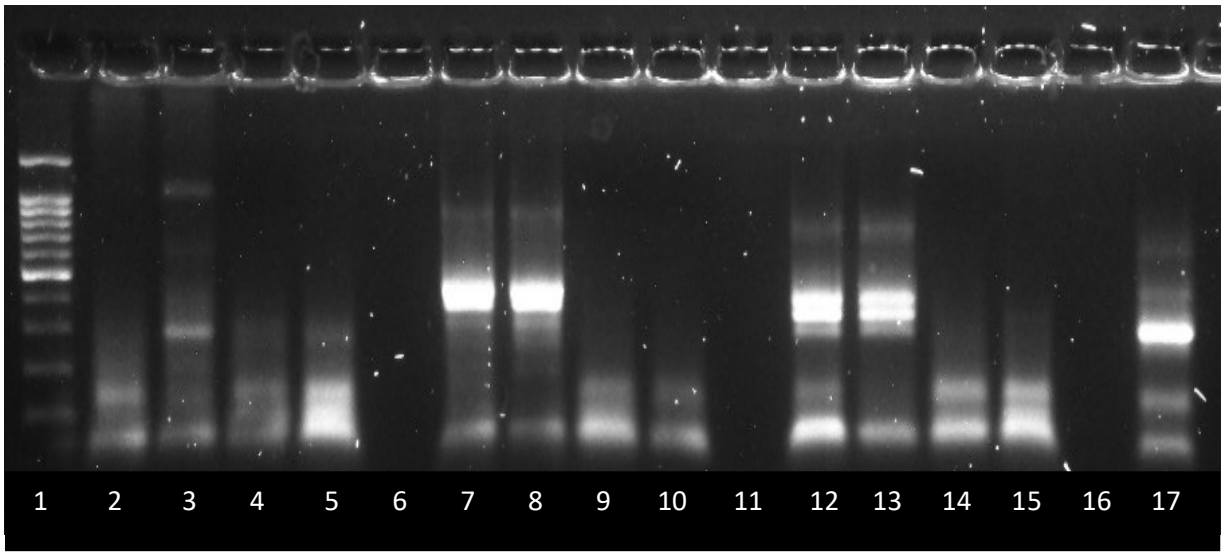

Figure 9: Picture showing results of a gel electrophoresis. Line 1 shows a 100bp marker. Line 17 is the positive marker for Candida spp. Lines 7, 8 and 12, 13 (duplicates) show positive samples (lanes 4, 8 and 14, 15 are the corresponding no template controls in duplicate of the extraction kit)

2.3.2 Sequencing

Amplicons from up to 60 samples were pooled equimolarly and subjected to emulsion PCR using the Ion 530 Chef Kit and the 400bp workflow according to manufacturer's protocols. After emulsion PCR the beads are loaded onto Ion Torrent 530 chips for sequencing. Sequencing reactions were performed on Ion Torrent S5XL using the Ion 400bp Sequencing Kit running for 1000 flows (all reagents from *Thermo Fisher Scientific,* MA, USA). Sequences were split by barcode and transferred to the *Torrent Suite* server. Unmapped bam files were used as input for bioinformatics.

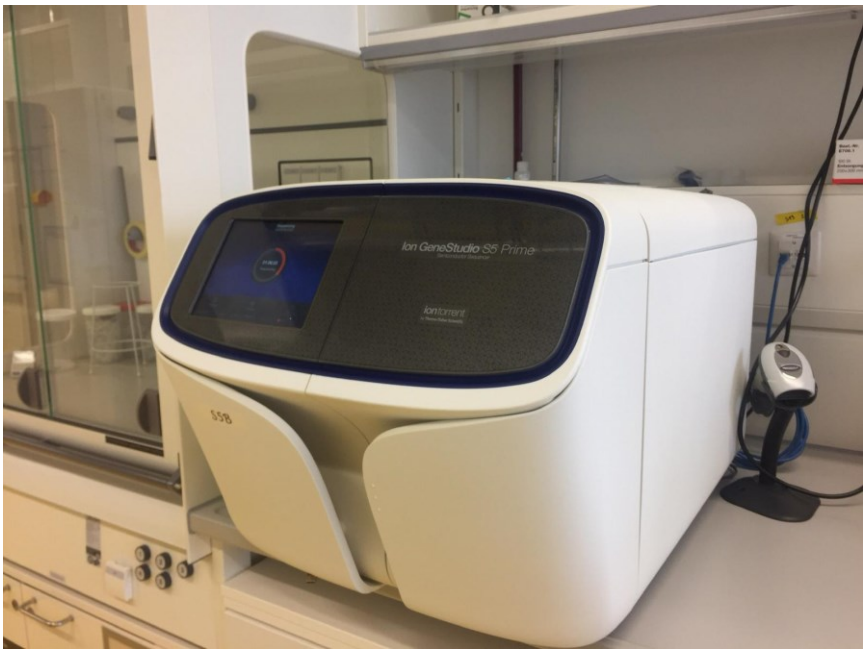

Figure 10: Ion Torrent S5XL used in the lab of the Medical University of Graz. After emulsion PCR the beads are loaded onto Ion Torrent 530 chips for sequencing.

2.3.3 Bioinformatics and Phylogenic Analysis

All sequences were initially trimmed by a sliding window quality filter with a width of 20nt and a cut off of Q20. Reads shorter than 100 nucleotides and reads mapping to the human genome were removed using deconseq (72). The resulting reads were subjected to error correction using the Acacia tool (73) leading to error correction of 10-20% of reads. Subsequently PCR chimeras were removed by use arch algorithm in de-novo and reference based settings (74). The final sequence files were then analysed by QIIME 1.8 workflow scripts (75). OTU search was performed using the parallel_pick_open_reference_otus workflow script and the Unite 12_11 reference database.

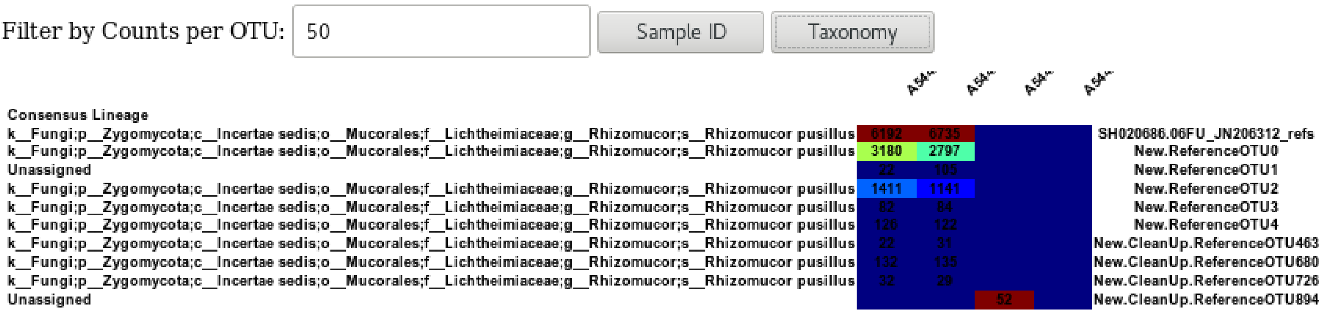

Figure 11: Picture showing the result of an ITS PCR and sequencing in the parallel_pick_open_reference_otus workflow script; the identified fungi belongs to the order of mucorales, specified as Rhizomucor pusillus.

2.3.4 Statistical analysis and visualization

OTUs were visualized as OTU tables, bar charts and PCOA plots using the Qiime core microbiome script. Additionally, groupings supplied in the mapping file were tested for statistical significance using the QIIME implementation of the Adonis test and significance of individual fungal strains was determined by Kruskal-Wallis test. Lefse (76) analysis was performed to detect statistically relevant strains in several of the study groupings.

3 Results

3.1 Demographic data

Of all patients, 36.6% were female (n=26) and 63.4% were male (n=45). The average age was 47 years and the median age was 54 years. The youngest patient was 0 years old and the oldest patient was 84 years old.

82% of the patients had an underlying disease. Solid organ cancer was the most common underlying disease at 24% (n=17). Seventeen percent had hematologic malignancies as an underlying condition (n=12), 10% suffered from congenital disorders (n=7), 7% had diabetes mellitus (n=5), 7% were solid organ transplant recipients (n=5), 1% had stem cell transplantation (n=1). Thirty-five percent were suffering from other underlying diseases that have not been considered risk factors for invasive fungal infections (n=25). Eighteen percent lacked any underlying disease (n=13).

Seventy-seven percent of the patients survived (n=55) while 22.5% died (n=16) during the hospital stay, but in only two cases the mycosis was considered the main cause of death.

3.2 Clinical sample selection

In total, 88 samples of 78 patients were analysed for this study (figure 9). Seven patients had to be excluded due to insufficient data documentation. Thus, 81 samples of 71 patients (four patients had two samples and three patients had three samples) were included. The specimen included: lung tissue (n=21), FFPE skin (n=9), bronchoalveolar lavage fluid (n=8), eye swab (n=5), liver tissue (n=4), sinus tissue or swab (n=4), pleural fluid (n=4), stool (n=3), liquor (n=3), sputum (n=2) and others (n=18), including two aortic valves, two maxillary sinuses, two tissue from the middle ear, two lymph nodes, two tissue of bones, one retroperitoneal tissue, one otorrhoea swab, one omentum tissue, one lumbar vertebrae, one brain abscess, one pericardium tissue and one intervertebral disc tissue.

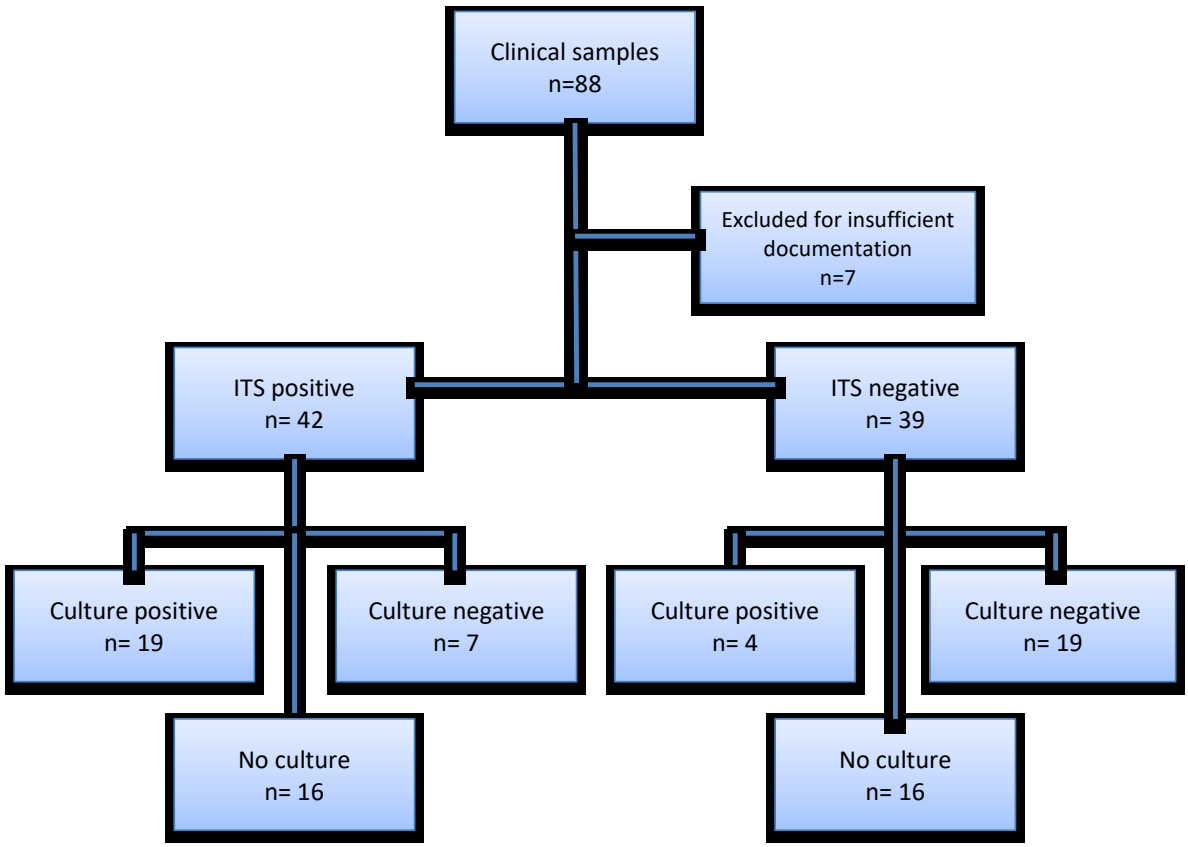

Figure 12: The selection process of sufficient specimen

3.3 Comparison of the results

ITS PCR and sequencing was positive in 51.9% (n=42) and negative in 48.1% (n=39) of the times (figure 12).

According to the *EORTC/MSG* criteria, nine of the patients (13%) had “proven” invasive fungal disease while ten (15%) could have been assigned to the category “probable”. The samples of five patients (7%) suggested that they had “possible” fungal disease. Out of the 69 patients, 45 patients (65%) had no invasive fungal infection at the point of sampling according to *the EORTC/MSG*-criteria for IFI. In two patients data was insufficient for classification (3),.

In total, 74 imaging procedures were done in the 71 patients. Pathological morphologies were found in 50 investigations (68%).

Microbiological culture of specimens obtained from the same body area as the material taken for ITS PCR and sequencing was positive in 28.4% (n=23) and negative in 32.1% (n=26) of the times. In 39.5% (n=32) either no culture was performed or the culture was from a different specimen not related to the specimen used for ITS PCR and sequencing. The reasons for omitting cultures in these particular cases were not specified in patient charts.

The concordance detected between ITS PCR and culture results was 46.9%. In 23.5% (n=19) of the results were PCR and culture positive and 23.5% (n=19) of the results were PCR and culture negative. Discordant results were found in 13.5% of the cases. 8.6% (n=7) of the cases were PCR positive and culture negative and 4.9% (n=4) of the cases were PCR negative and culture positive. The missing 39.5% can be attributed to those cases where no culture was done.
If the cases in which no culture was done would be excluded, the found concordance between ITS PCR and culture would be 77.6%. 38.8% of ITS PCR and culture results would be positive and negative respectively. The discordance would be 22.4% with 14.3% ITS PCR positive and culture negative and 8.2% ITS PCR negative and culture positive results (table 9).

Table 9: Concordance of ITS PCR and culture, when cases whithout culture are excluded; found concordance was 77.6%. Abbreviations: - = negative, + = positive

		Culture	
		+	-
ITSPCR	+	38,8%	14,3%
	-	8,2%	38,8%

In 23.5% (n=19) of all cases with positive results in both culture and ITS PCR, the organisms identified were not always the same. In 14 of the samples the same fungus was identified (table 10). In 10 of these samples the identified fungus was assigned to Candida species. Even though ITS sequencing as well as culture were positive and identified the same fungi, according to the *EORTC/MSG* criteria, 7 of the patients had no IFI at all.

In 12 out of the 14 cases where the same fungi were found in ITS sequencing and culture, the cultural results were available before the ITS sequencing results. In only one sample the result of the ITS sequencing was available one day earlier than the result of the culture, and once the results of the culture and those of the ITS PCR and sequencing were available on the same day. In two of these 14 cases the positive sequencing result lead to a modification in therapy.

Table 10: Samples with positive results of both ITS PCR and sequencing and culture and with identification of the same fungal genus or species

Specimen number	Clinical specimen	Fungal species identified by ITSPCR	Fungal species identified in culture	EORTC/MSG criteria	Date of positive ITSPCR	Date of positive culture	Therapy modification
3	lung tissue	Candida intermedia, Aspergillus pseudoglaucus	Candida krusei, Candida guiliermondii, Aspergillus fumigatus	No IFI	23.06.2015	01.06.2015	no
6	lung tissue	Rhizopus microsporus	Rhizopus microsporus	Proven IFI	06.08.2015	13.07.2015	yes
8	Stool	Candida albicans	Candida albicans	Probable IFI	16.11.2015	10.03.2015	no
12	retroperitoneal tissue	Candida albicans	Candida albicans	No IFI	14.01.2016	16.12.2015	no
14	omentum tissue	Candida albicans	Candida albicans	Proven IFI	25.07.2015	21.06.2015	no
16	Stool	Candida parapsilosis	Candida glabrata	No IFI	17.10.2016	19.09.2016	no
21	BAL fluid	Candida parapsilosis	Aspergillus fumigatus, Candida parapsilosis	No IFI	15.12.2016	15.12.2016	yes
28	liver tissue	Candida albicans	Candida albicans	Proven IFI	07.04.2017	24.03.2017	no
29.2	Sputum	Candida albicans	Candida albicans	Probable IFI	23.06.2017	24.06.2017	no information
46	aortic valve prothesis	Aspergillus terreus	Aspergillus terreus	Proven IFI	16.03.2018	13.03.2018	no
47	skin tissue	Aspergillus fumigatus	Aspergillus fumigatus	No IFI	21.03.2018	02.02.2018	no
54.1	lung tissue	Candida albicans, Cladosporium cladosporioides	Aspergillus fumigatus, Candida albicans	Proven IFI	04.07.2018	25.06.2018	no
56	Sputum	Candida albicans, Candida parapsilosis	Candida albicans, Aspergillus calidoustus	No IFI	01.08.2018	13.07.2018	no
70	Eye	Fusarium verticilloides/ proliferatum	Fusarium verticilloides/ proliferatum	No IFI	16.11.2018	14.11.2018	no

In 5 of the 19 samples with both ITS sequencing and culture positive results, ITS sequencing and culture identified different fungi (table 11). None of the fungi identified in the ITS sequencing were assigned to Candida spp. In contrast, three of the culture results showed Candida spp. None of the ITS sequencing results led to a modification of therapy. The reasons for the lack of a change in therapy were as follows: In one patient, the therapy has been modified after the culture results. In the second patient the ITS PCR and sequencing results was available after the patient's death. In another patient, the identified organism was considered a contaminant. In two other cases the exact reason for absent modification was not documented.

Table 11: Samples with positive results in both ITS PCR and culture but with identification of different fungal species

Specimen number	Clinical specimen	Fungal species identified by ITSPCR	Fungal species identified in culture	EORTC/MSG criteria	Therapy modification
2,2	lung tissue	Actinomucor elegans	Aspergillus fumigatus	Probable IFI	no
11	skin tissue	Cladosporium cladosporioides	Scendosporium apiospermum	No IFI	no
41	maxillary sinus tissue	Aspergillus fumigatus	Candida albicans	Probable IFI	no
45	skin tissue	Rhizopus microsporus	Candida glabrata	Probable IFI	no
64	lung tissue	Epicoccum nigrum	Candida albicans	Probable IFI	no

In seven cases (8.6%) the ITS PCR and sequencing was positive while the culture showed no fungal growth (table 12). Fungal elements could be identified in histology in all of the seven samples, but according to the *EORTC/MSG* criteria, five of the patients hat no IFI. Twice, the positive ITS PCR and sequencing result led to a modification in therapy. In one case the existing therapy was prolonged, in the second case the patient received a completely new antifungal agent. In the remaining five cases, the therapy was not changed after the ITS PCR and sequencing result: In one case, the results were only available post mortem. In two cases it was not clear why the therapy was not modified after the results showed a fungal organism. On one occasion the therapy has been stopped because of strong side effects before the results were available. Further, in one case the identified fungus was considered a colonizer.

Table 12: Samples with positive ITS PCR and sequencing and negative culture results

Specimen number	Clinical specime	Underlying disease	Fungal species identified by ITSPCR	Result in microscopy	EORTC/MSG criteria	Therapy modification
5	lung tissue	solide tumor	Cladosporium cladosporioides	positive	No IFI	yes
9	lung tissue	ANCA associated vasculitis	Cladosporium cladosporioides	positive	Proven IFI	no
10	BAL fluid	chronic granulomatous disease	Kodamaea ohmeri	positive	Proven IFI	no
18	liver tissue	solide tumor, SOT	Alternaria alternata	positive	No IFI	yes
19	lymph node	none	Fusarium equiseti, Epicoccum nigrum, Aspergillus caesiellus, Wallemia sebi	positive	No IFI	no
50	eye	none	Candida albicans	positive	No IFI	no
53	maxillary sinus tissue	solide tumor	Aspergillus fumigatus	positive	No IFI	no

In 5% (n=4) of the cases, the ITS PCR and sequencing was negative while the culture detected fungal growth (table 13). In each of these cultures, Candida spp. was one of the identified fungal species. Therapy has been modified once. The negative ITS sequencing led to the discontinuation of the antifungal agent. In two cases the therapy has not been modified after the availability of negative ITS sequencing results. In these cases, the patients did not get any antifungal medication anyway. In one occasion, the therapy has been modified after the positive culture and the ITS sequencing result did not change that.

Table 13: Samples with negative ITS PCR and sequencing and positive culture results

Specimen number	Clinical specimen for ISTSPCR	Clinical specimen for culture	Underlying disease	Fungal species identified by culture	Result in microscopy	EORTC/MSG criteria	Therapy modification
1	lung tissue	Sputum	Mucoviscidosis	Candida Krusei, Candida parapsilosis, Candida famata	no microscopy	No IFI	no
2,1	BAL fluid	BAL, Sputum	Hematologic malignancies	Aspergillus fumigatus, Candida albicans	positive	Probable IFI	no
40	lung tissue	BAL	Solid tumor	Candida albicans	no microscopy	Probable IFI	yes
54,2	BAL fluid	BAL, Sputum	Mounier-Kühn syndrome	Aspergillus fumigatus, Candida albicans	no microscopy	Proven IFI	no

3.4 Infectious diseases consultation service

ITS PCR and sequencing was ordered by infectious disease consultants for 29 (36%) of samples, by pathologists for 16 (20%), and by others for 36 (44%) of samples.

3.5 Therapy modification

Overall, ITS sequencing results informed antifungal treatment strategy in 64/71 (90%) of patients. In 16 patients (23%) with positive ITS sequencing results and missing cultures, therapy was modified in 11/16 patients (69%) and considered as already targeted treatment addressing the identified fungus in 2/16 patients (13%). In 60 patients (85%) antifungal treatment strategy remained unchanged after receipt of ITS sequencing due to the following reasons: ITS sequencing remained negative and there was no need for antifungal therapy in 26/60 patients (43%); ITS sequencing result interpreted as contamination in 9/60 (15%); adequate antifungal therapy already ongoing without need for modification in 17/60 (28%); and ITS sequencing results obtained post-mortem in 7/60 (12%). In one patient with positive ITS sequencing result of a corneal sample local antifungal therapy was initiated but no systemic antifungal treatment (table 14).

Table 14: Cases with therapy modification after the ITS PCR and sequencing results were available. Additionally, the comparison of ITS PCR and sequencing results to culture and *EORTC/MSG* criteria are shown. The patient with specimen number 32 was transferred to another hospital before therapy modification.

Specimen number	Type of modification	ITSPCR resu	culture resu	EORTC/MSG criteria
5	prolongation Posaconazol	Cladosporium cladosporioides	negative	No IFI
6	stop antibiotic treatment, Posaconazol as tablet	Rhizopus microsporus	Rhizopus microsporus	Proven IFI
15	Anidulafungin	Candida albicans	no culture done	Possible IFI
18	Anidulafungin	Alternaria alternata	negative	No IFI
20	Voriconazol	Aspergillus fumigatus	no culture done	Probable IFI
21	Caspofungin	Candida parapsilosis	Aspergillus fumigatus, Candida parapsilosis	No IFI
27	discontinue Posaconazol, start Voriconazol	Aspergillus fumigatus	no culture done	No IFI
32		Geomyces pannorum, Naevala minutissima	no culture done	Proven IFI
37	Isavuconazol	Aspergillus fumigatus	no culture done	-
40	stop Voriconazol	negative	Candida albicans	Probable IFI
55,1	stop antifungal treatment	negative	negative	Probable IFI
55,2	stop antifungal treatment	negative	negative	Probable IFI
62,1	Trimethoprim/ Sulfamethoxazol Solution	Pneumocystis	no culture done	No IFI

3.6 Case study one

The patient is F.S. who had mucormycosis in year 2015 when he was 60 years old. F.S. suffered from secondary acute myeloid leukaemia developed from myelodysplastic syndrome. In 2010 he received allogenic stem cell transplantation and immunosuppression. Starting in 2011, he additionally received glucocorticoid therapy due to severe GVHD. As a side effect he developed steroid induced diabetes mellitus.

In June 2015, F.S. was admitted to hospital due to dyspnoea with the suspicion of pulmonary embolism. The CT scan showed a cavern in the left inferior lobe of the lung with perifocal ground-glass infiltration of lung parenchyma. Bacterial and mycobacterial etiologies were ruled out by bronchoscopy and subsequent culture and TB PCR of samples obtained from the lower respiratory tract. Due to the underlying disease and suspicion of fungal infection liposomal amphotericin B was started.

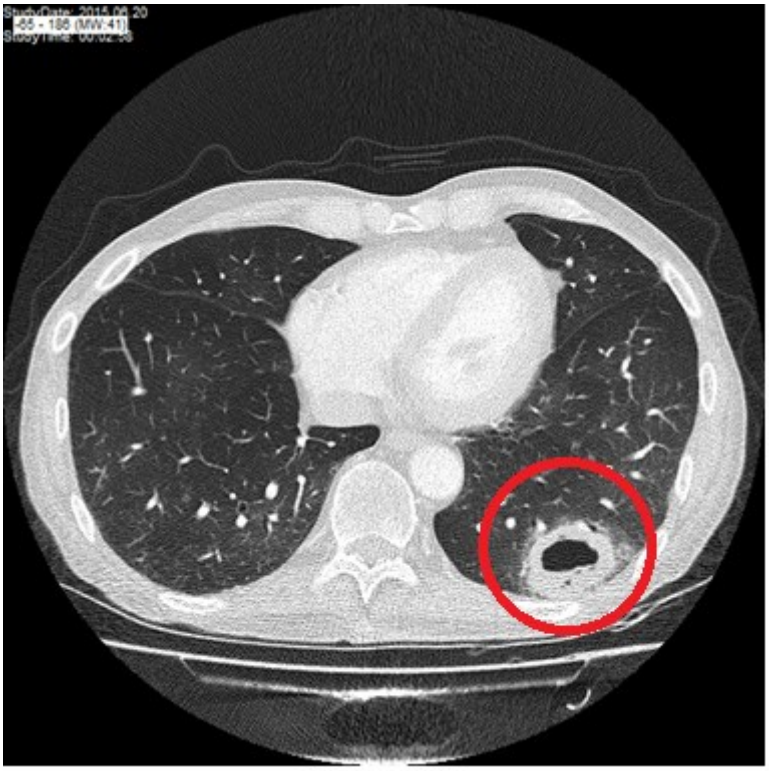

Figure 13: CT scan of the lung of F.S. from the 06/20/2015 showing a cavernous lesion in the left inferior lobe of the lung (red circle) with perifocal ground-glass infiltration of the lung parenchyma.

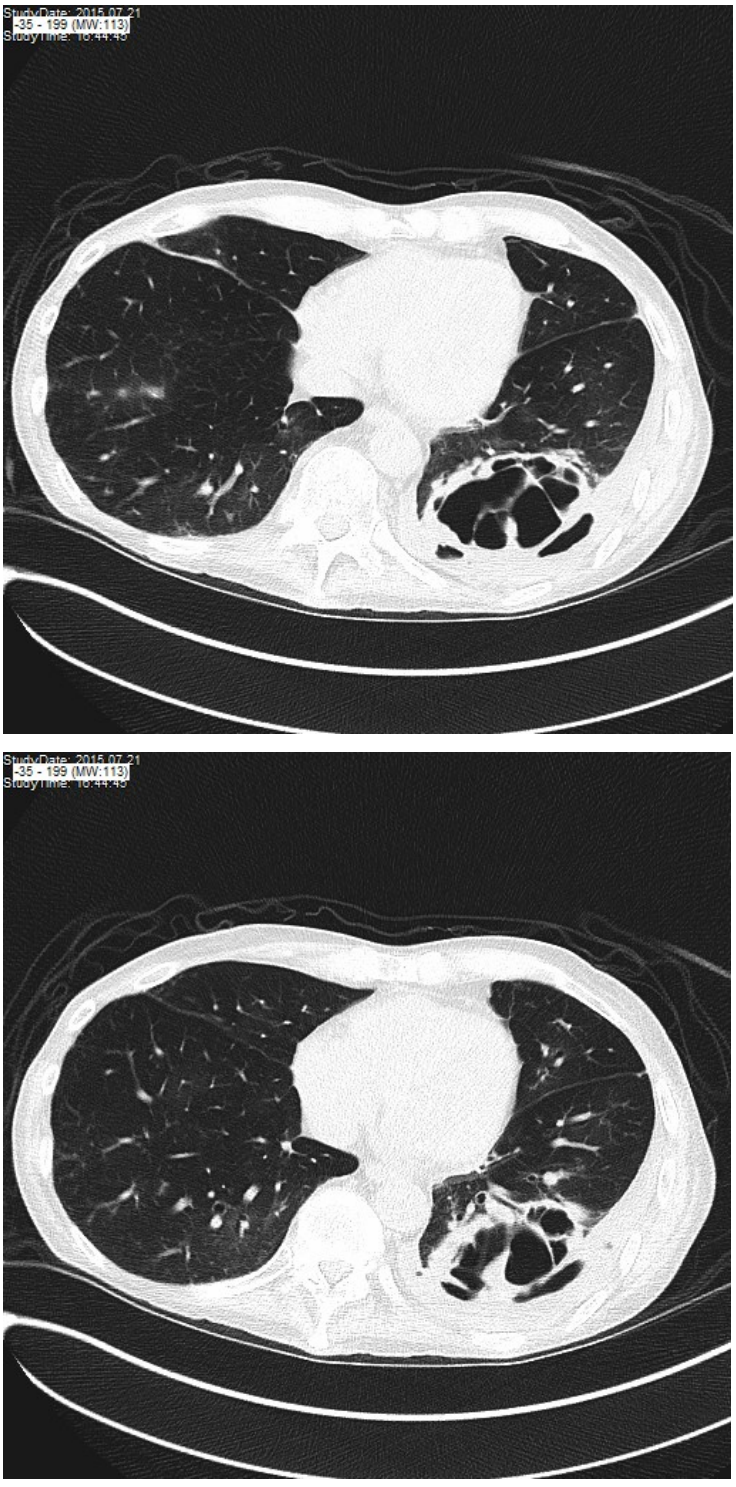

Figure 14: Two different slices of the chest CT scan of patient F.S. approximately one month after the initial CT scan above, showing a progression of the cavernous lesion

Afterwards, the result of the sputum showed the growth of *Rhizopus microsporus*. Spontaneous rupture of the cavernous lesion and subsequent pneumothorax led to

resection of the lower left lobe. Microscopy of the resected tissue showed a circumscribed invasive “aspergilloma”, and, in the part of the resected diaphragm, fungal hyphae and spores were found. Intra- and post-surgically obtained swabs of pleural fluid did not yield evidence of fungal growth. GM and BDG were negative. Subsequently, an infectious disease consultant ordered ITS PCR and sequencing of the resected tissue. The results of the ITS PCR and sequencing confirmed the culture results showing Rhizopus microsporus. Until this date, the patient received liposomal amphotericin B and meropenem. During this therapy, the elevated levels of inflammatory parameters decreased and the patient was clinically stable. Following a recommendation of the infectious disease consultant the therapy with liposomal amphotericin B and meropenem was discontinued and replaced by posaconazol 300mg tablet.

On the 08/24/2015, F.S. was discharged from hospital into home care.

3.7 Case study two

The patient for this case study is J.M., a 11-year-old girl suffering from cystic fibrosis. She was admitted to the hospital because of tachydyspnea with a respiratory rate of 32/minute, cough and haemoptysis and a bad overall physical constitution. Due to her decreasing health condition, five days prior to admission, a bronchoalveolar lavage was performed. In a CT scan one day before the BAL, several consolidations in the lingual, the inferior lobe and the superior lobe on both sides of the lung were visible and described as a tree-in-bud phenomenon.

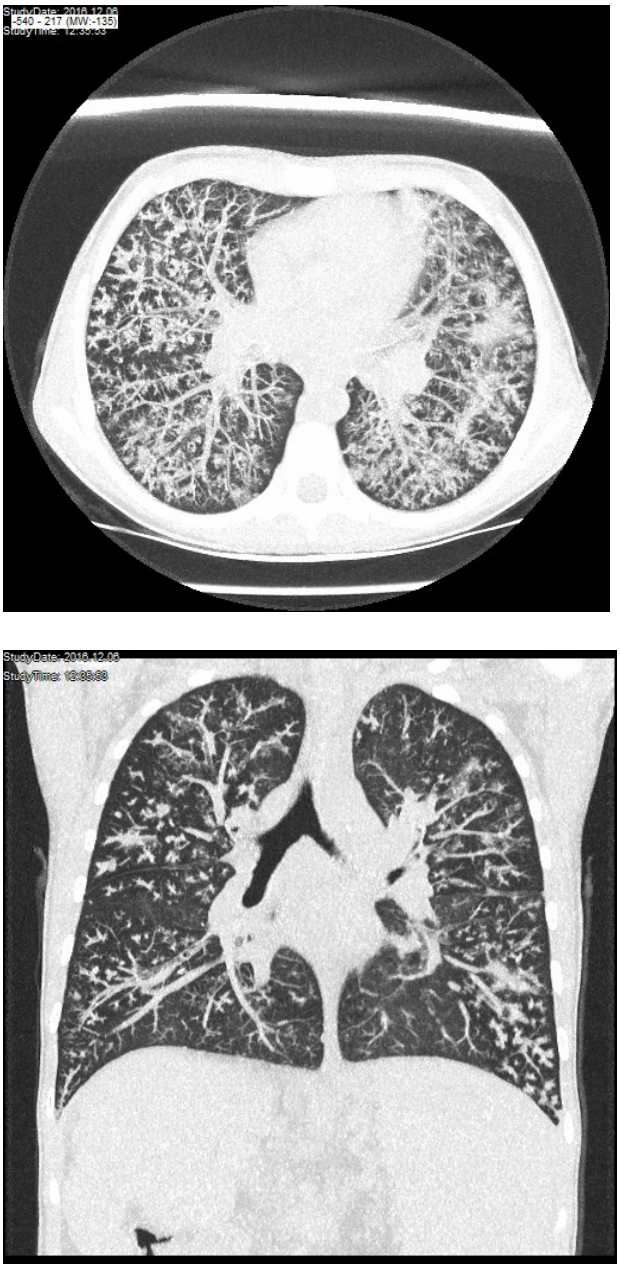

Figure 15: CT scan of the lung of J.M. from the 12/06/2016 showing typical tree-in-a-bud structure

The BAL fluid was sent to the pathology department. The microscopy revealed a neutrophil alveolitis with the detection of hyphae. Consequently, the pathologists initiated the ITS PCR and sequencing of the BAL fluid. The results were available on the same day as the cultural results of the BAL fluid, and they both identified *Candida parapsilosis*. Although Candida species in the lower respiratory tract is usually considered a colonizer, the treating physicians initiated caspofungin due to the underlying condition, as well as ceftazidime, amikacine and teicoplanin. The patients responded to antiinfective therapy and on the 12/31/2016, J.M. was discharged from hospital into home care.

4 Discussion

Fungal infections are a main cause of morbidity and mortality in different patient groups. Despite new antifungal drugs and some new diagnostic procedures, the mortality rate of fungal infections remains high. One reason therefore is the challenge of the diagnostic process and early administration of antifungal therapy (2). The aim of this study was to investigate the impact of ITS PCR and sequencing on antifungal treatment of patients suffering from invasive fungal infections. For that, data of 71 patients with 81 samples overall was analysed, and results from ITS PCR and sequencing and standard fungal culture were compared. It was further analysed whether the results led to initiation, discontinuation or an adjustment of therapy. Special attention was turned to the cases with negative culture results and positive ITS PCR and sequencing. Additionally, it was investigated whether clinicians would rather rely on results of fungal culture or ITS sequencing for the further treatment. Furthermore, all results were compared with the clinical presentation of the patients, which was characterised with the *EORTC/MSG* criteria, and the results of the microscopy to find out whether a fungal infection was existent.

The concordance between ITS PCR and sequencing and culture was 77.6%, This means that in 19 out of 81 cases, culture as well as ITS PCR indicated the presence of a fungal pathogen. In 14 of these 19 cases, the identified fungal genus, and in 12 cases even the identified fungal species were the same. In only 2 of the 19 cases the results of the ITS PCR led to a modification in therapy. In most cases, the therapy was mostly retained because of the delayed availability of ITS PCR results. On average, the results of ITS PCR and sequencing were available one month after the results of the culture. In most cases, the therapy was modified according to the culture results, and ITS PCR only confirmed them. The latter is, however, very important in terms of potential side effects, costs and outcome.

In their study in 2016, *Rampini et al*. compared the clinical impact of ITS PCR and sequencing with a culture in non-immunocompromised patients. They found a high concordance of 89.6% in the results for ITS PCR and sequencing and culture. The authors mentioned, that the advantages of ITS PCR were the fast performance of the method and its ability to identify even rare fungal organisms to a species level (70).

The results of *Rampini et al*. suggest that the long turnaround time found in our analysis of ITS sequencing is not a problem of the ITS PCR and sequencing itself, but maybe due to organisational and procedural aspects of this technique in our centre. During the 4 years of ITS PCR and sequencing results performed in our pathology laboratory there was an improvement in turnaround time as well as technical and personal aspects. As a consequence, ITS PCR and sequencing should be at least as fast as other diagnostic procedures as timing of antifungal therapy critical point in the treatment of invasive fungal infections. Hence, the antifungal treatment has to be implemented as soon as possible to improve the outcome of the patients.

Due to the high concordance of ITS PCR and sequencing and fungal culture, doing both methods increases the probability of diagnosing fungal infections only minimally, but the confirmation strengthens the choice of antifungal treatment strategies. In 7 cases, the cultural results were negative whereas the ITS sequencing brought positive results. In all of these cases, fungal elements were found during microscopy, which indicates that a fungal infection was existent in these patients. Interestingly, according to the *EORTC/MSG* criteria, 5 of the 7 cases had “no fungal infection”, and only in two cases the infection was “proven”. This number is too low to evaluate the diagnostic value of ITS PCR in culture negative fungal infections, but it illustrates the difficulties in diagnosing them. The solution has to be an interdisciplinary approach, where different aspects, such as the clinical presentation, radiological findings, laboratory results and molecular methods like ITS PCR are included.

Pathogens that cause IFI in humans are shifting from *Candida albicans* species to non-*Candida-albicans* and from Aspergillus to non-Aspergillus species (12). In this study, most of the fungi identified with ITS sequencing, did not belong to the species Candida or Aspergillus. This was particularly the case when the culture was negative and ITS PCR brought positive results and in the cases where the identified fungi in culture and ITS PCR differed. In the culture-negative-PCR-positive group, the identified fungi were *Cladosporium cladosporioides* (n=2), *Kodamaea ohmeri* (n=1), *Alternaria alternate* (n=1), *Fusarium equiseti, Epicoccum nigrum, Aspergillus caesiellus, Wallemia sebi* (all in one specimen, n=1).

In the group with different ITS PCR and sequencing and culture results, the fungi identified in culture belonged to Candida spp. (n=3), Aspergillus spp. (n=1) and

Scendosporium apiospermum (n=1). The fungi identified in ITS PCR and sequencing belonged only once to Aspergillus spp., but also *Actinomucor elegans* (n=1), *Cladosporium cladosporioides* (n=1), *Epicoccum nigrum* (n=1) and *Rhizopus microsporus* (n=1). This maybe illustrates that culture is not suitable for the identification of non-Candida and non-Aspergillus species fungi.
Hammond et al. confirmed that ITS PCR and sequencing is a useful and reliable tool in the diagnosis of mucormycosis. It is especially useful with a negative fungal culture, to identify the fungi to a species level and thereby making targeted antifungal treatment possible (77).

One problem that occurred was the missing culture in 39.5% (n=32) and microscopy in 35.8% (n=29) of the patients. In only 35 samples both microscopy and culture were done in addition to the ITS PCR and sequencing. In 50% of the cases (n=16) with missing cultures the ITS sequencing showed a positive result. In six of these 16 cases, therapy was modified according to the ITS PCR results. It would have been interesting to know if the culture had shown fungi too, or if these infections could have been identified solely in the molecular essay. In other four of these 16 cases, the identified fungi in the ITS PCR and sequencing were categorized as contamination.

4.1 Strengths and limitations

The strength of this study was that it was the first study addressing ITS PCR and sequencing in clinical context including the clinical impact of its results. Other studies, e.g. published by *Lass-Flörl et al.,* compared the results of ITS sequencing with fungal culture and microscopy and analysed the patients in a clinical context, but did not investigate the clinical impact of the method (12). We consider this element of the study the most important one, as it shows whether implementation of ITS PCR and sequencing has an effect on treatment of patients. In our study, ITS PCR and sequencing had an impact especially in those cases with a remaining negative culture.

There are several limitations of this study, however. For once, only specimen of 71 patients were analysed. To profoundly assess the effect of ITS sequencing on patient outcome, data of more patients must be evaluated and compared to the outcome in

patients where no ITS PCR was used for diagnosis. Another limitation is the insufficient data documentation. In 30.9% of the cases, it is not clear who initiated the ITS PCR and sequencing method. It would be important to know if the method is only known by the infectious disease specialists, in which case the knowledge of this tool needs to be forwarded to the other departments in order to let the ITS PCR become a standard method in the diagnostic process of fungal infections.

The main shortcoming is the missing culture in a lot of cases. The reasons for missing cultures in 40% of samples remained unclear but maybe due to local procedures as cultures and ITS-sequencing were performed in two distant laboratories (cultures in the microbiolgical laboratory and ITS sequencing in the pathology laboratory). Clinicians therefore had to send two adjacent samples to each of the labs. In 26% of samples undergoing combined ITS sequencing and cultures, the investigations showed discordant results. ITS sequencing might therefore provide additional information in patients with difficult to culture fungi or in patients already receiving antifungal treatment prior to sampling which might influence fungal growth. In seven patients, ITS-sequencing results were obtained postmortem. Time-to result is a critical issue for every diagnostic tool especially in rapidly progressive fungal diseases like mucormycosis (56). As actually no fungal biomarker can be used for anticipation of mucormycosis, faster sequencing technologies of tissues or other samples may prospectively be useful in earlier diagnosis of this often fatal disease (56).

4.2 Conclusion

ITS PCR and sequencing is a promising method to diagnose fungal infections, especially when they are caused by non-Aspergillus and non-Candida species. In conclusion, our study found that ITS sequencing, despite a long turnaround time, informed antifungal treatment strategies in 90% of patients. ITS sequencing confirmed already ongoing antifungal therapy (19/71 patients, 27%), led to change of antifungal therapy (11/71 patients, 15%) or supported the decision to withhold antifungal treatment (34/71 patients, 48%). Owing to the current difficulties in elucidating the fungal etiology and subsequently selecting the correct antifungal treatment we need further data of modern molecular based technologies including

metagenomic shot-gun long read sequencing for identification of pathogenic fungal species in patients with IFIs.

IFI will be a crucial issue in the future, and the diagnostic process has to be interdisciplinary with the ITS PCR as one of the pillars to grand the best possible treatment for the patients.

5 Literature

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