

Diploma Thesis

**Comparison of different diagnostic criteria for invasive
aspergillosis in regard to epidemiology and outcome of patients**

submitted by

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Graz, 17.09.2019

Declaration

I declare that I have written this diploma thesis independently, that I have not used other than the sources/resources cited, and that I have explicitly marked all material which has been quoted either literally or by content from the sources used.

Graz, 17.09.2019

Clemens Renhart, e.h.

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1. List of Abbreviations

ABPA = allergic bronchopulmonal aspergillosis

AIDS = acquired immunodeficiency syndrome

AmB = Amphotericin B

AML = acute myeloid leukaemia

ARDS = acute respiratory distress syndrome

BAL = bronchoalveolar lavage

BALF = bronchoalveolar lavage fluid

CABG = coronary artery bypass graft

CCPA = chronic cavitary pulmonal aspergillosis

CMV = cytomegaly virus

CNS = central nervous system

COPD = chronic obstructive pulmonary disease

CSF = cerebrospinal fluid

CT = computed tomography

DIC = disseminated intravascular coagulation

EBA = Erstuntersuchung-Beobachtung-Aufnahme (Emergency Room)

ECIL = European Conference on Infections in Leukaemia

ECMO = extracorporeal membrane oxygenation

EIA = enzyme linked immunoassay

EMA = European Medicines Agency

ENT = Ear, Nose and Throat

EORTC/MSG = European Organization for Research and Treatment of Cancer/Mycoses Study Group

ESCMID = European Society of Clinical Microbiology and Infectious Diseases

FDA = Food and Drug Administration

GM = galactomannan

G6PDD = glucose-6-phosphate dehydrogenase deficiency

HRCT = high resolution computed tomography

HSCT = hematopoietic stem cell transplantation

IA = invasive aspergillosis

IPA = invasive pulmonal aspergillosis

ICU = intensive care unit

IFI = invasive fungal infection

MRI = magnetic resonance imaging

NHL = Non-Hodgkin-Lymphoma

NPV = negative predictive value

ODI = optical density index

PMNL = polymorphonuclear lymphocytes

PPV = positive predictive value

PRR = pathogen recognising receptors

RCU = respiratory care unit

SOT = solid organ transplantation

TDM = therapeutic drug monitoring

TLR = toll like receptor

TNF- α = tumor necrosis factor α

UV = ultraviolet

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4. Abstract

4.1 Background

The European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) published criteria for diagnosing Invasive Aspergillosis (IA) in 2002 and 2008. There are also criteria for intensive care unit (ICU) patients without classic host factors. A single center retrospective study was performed to examine changes in epidemiology and outcome using the three criteria.

4.2 Methods

From October 2011 to October 2017, 899 samples of bronchoalveolar lavage fluid (BALF) from 617 patients were taken to conduct this study. Patient data were collected using the medical data system used by the KAGes network of hospitals in Styria, Austria. Patients were categorised according to the three diagnostic criteria. Frequencies were calculated, cumulative survival was calculated using Kaplan-Meier estimators.

4.3 Results

Of 617 patients, two had proven IA. According to the 2008 criteria, seven (1.1%) had probable IA compared to four (0.7%) using the 2002 criteria, three patients were non-classifiable due to missing host factors. Main underlying diseases were acute leukaemia and COPD. Four of 265 ICU patients had putative IA, two of them were non-classifiable using the 2008 criteria, due to missing host factors.

Using the EORTC/MSG 2008 criteria 13 (2.1%) had possible IA vs. 26 (4.2%) using the 2002 criteria. Eight of possible IA cases using the 2002 criteria had possible IA using the revised criteria, the rest was non-classifiable due to lack of host factors or CT signs. Seven (1.1%) patients had CT signs and mycological criteria, but no host factors. Main underlying diseases were liver cirrhosis and COPD.

4.4 Conclusions

Both proven IA cases were diagnosed *post mortem*, due to missing host factors or CT signs. Seven (1.1%) patients had probable IA. Two of four putative IA cases were missed with the EORTC criteria due to missing host factors. Possible cases were reduced, mostly

due to missing host factors and CT signs. Seven (1.1%) patients showed CT signs and mycological signs, but no host factors and were thus non-classifiable. Broadening the range of host factors (liver cirrhosis, new immunosuppressive agents, COPD, influenza) and using more or more specific mycological tests might prove useful to ensure diagnosis.

5. Zusammenfassung

5.1 Hintergrund

Die European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) veröffentlichte 2002 und 2008 Kriterien, um invasive Aspergillosen (IA) zu diagnostizieren. Ebenso gibt es Kriterien für intensivmedizinisch betreute PatientInnen ohne klassische Risikofaktoren. Eine monozentrische retrospektive Studie wurde durchgeführt, um die drei Kriterien bezüglich Veränderungen in Epidemiologie und Outcome zu vergleichen.

5.2 Methoden

Um diese Studie durchzuführen wurden zwischen Oktober 2011 und Oktober 2017 insgesamt 899 Proben von bronchoalveolärer Lavageflüssigkeit (BALF) von 617 PatientInnen untersucht. PatientInnenendaten wurden über das PatientInnenendatensystem der KAGes-Krankenhäuser in der Steiermark (Österreich) gesammelt. Die Datenanalyse wurde mittels IBM SPSS Statistics 25 (IBM Corporation, Armonk, NY) durchgeführt. Häufigkeiten wurden erhoben, kumulatives Überleben wurde mittels Kaplan-Meier-Schätzer berechnet.

5.3 Ergebnisse

Von 617 PatientInnen hatten zwei eine bewiesene IA. Gemäß den Kriterien 2008 hatten sieben (1.1%) eine wahrscheinliche IA verglichen mit vier (0.7%) nach den Kriterien von 2002. Acht der möglichen IA Fälle gemäß den 2002 Kriterien hatten auch mit den aktualisierten Kriterien eine mögliche IA, der Rest war nicht klassifizierbar aufgrund fehlender Risikofaktoren oder CT-Zeichen. Sieben (1.1%) PatientInnen hatten CT-Zeichen und mykologische Kriterien, aber keine Risikofaktoren. Wichtige zugrundeliegende Krankheiten waren hierbei Leberzirrhose und COPD.

5.4 Konklusion

Beide bewiesenen IA-Fälle wurden *post mortem* diagnostiziert, aufgrund fehlender Risikofaktoren oder CT-Zeichen. Sieben (1.1%) PatientInnen hatten wahrscheinliche IA. Zwei von vier mutmaßlichen IA-Fällen wurden mit den EORTC-Kriterien nicht diagnostiziert, aufgrund fehlender Risikofaktoren. Mögliche IA-Fälle wurden reduziert,

zumeist aufgrund fehlender Risikofaktoren und CT-Zeichen. Sieben (1.1%) PatientInnen zeigten CT-Zeichen und mykologische Zeichen, aber keine Risikofaktoren und waren dadurch nicht klassifizierbar. Eine Erweiterung der Risikofaktoren (Leberzirrhose, neue Immunsuppressiva, COPD, Influenza) und Anwendung von mehr oder spezifischeren mykologischen Tests könnte sich als nützlich erweisen um die Diagnose zu sichern.

6. Introduction

6.1 Characterisation of *Aspergillus fumigatus*

6.1.1 Biology and distribution

Aspergillus is a saprophytic mould that is forming mycelia. As a decomposer it is living in soil, decomposing organic matter, seeds and grains. The genus can be split in 22 different sections, of which ten contain clinically relevant species (*Aspergillus*, *Fumigati*, *Circumdati*, *Terrei*, *Nidulantes*, *Ornati*, *Warcupi*, *Candidi*, *Restricti*, *Usti*, *Flavipedes*, and *Versicolores*)⁽¹⁾. Recent phylogenetic studies imply that *Aspergillus* is a monophyletic genus, closely related to *Penicillium*⁽²⁾.

Aspergillus fumigatus can be isolated in any season from a large variety of substrates. Examinations of garden soil and greenhouses showed that *Aspergillus* made up between 35 and 70% of all colony building fungi on these substrates⁽³⁾. The species is impressively versatile and adaptable. Although best growth is to be seen at a temperature of 37°C and a pH between 3.7 and 7.6, *Aspergillus* can be found at any place with a substrate at a temperature between 12°C and 65°C, and a pH between 2.1 and 8.8. It has a versatile metabolism for utilising nutrients and various components of cell walls of plants^(3, 4).

6.1.2 Ways of reproduction

Aspergillus fumigatus mainly reproduces in an anamorph mode. Spores (conidia), which are built asexually, are distributed in the environment mainly through the air. When conidia find good conditions for growth, they start germinating and form a vegetative mycelium. Following that, the mycelium will form specialised organs carrying conidia (conidiophores), built to release the spores⁽⁵⁾. This is pictured in Figure 1.

Recent studies discovered a sexual cycle as well. When sequencing the genome, a large number of genes could be found that are typical for teleomorph fungi⁽⁶⁾. These results lead to problems concerning traditional taxonomy of *Aspergillus*: The anamorph form of *Aspergillus* is corresponding to the teleomorph form *Neosartorya*. Correctly, there must be differentiated between the asexual and sexual form. For reasons of simplicity, the denomination *Aspergillus* was chosen consensually⁽²⁾.

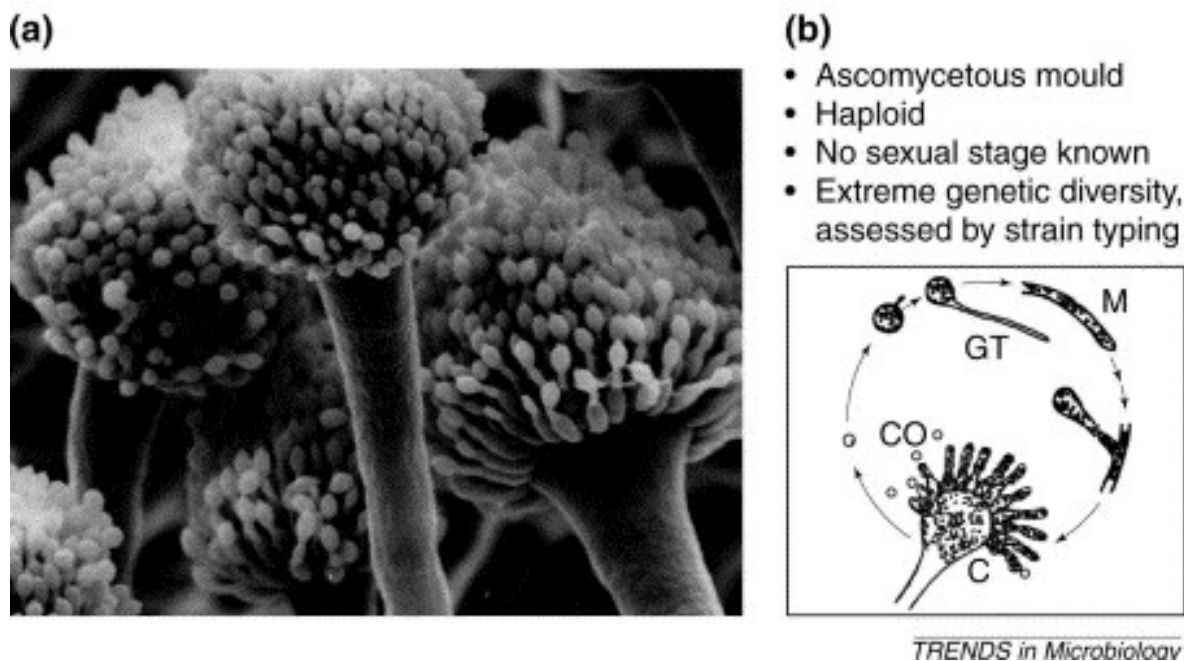


Figure 1. (a) Conidiophores with conidia (b) Reproduction cycle of *Aspergillus*. Reprinted from ⁽⁵⁾ with permission from Elsevier. Copyright ©2001 Elsevier.

6.1.3 Composition and structure

Cell walls as well as the membranes of each living organism have to perform various tasks. They are a protection against external influences, serve as a way of communication with the organism's environment, and are an important way to determine the pathogenicity. Furthermore, a solid understanding of the composition of these structures is of importance because several antifungal agents act by influencing structure or synthesis of the cell wall or the membrane. Certain components of the cell wall, for instance galactomannan (GM) or 1,3- β -D-glucan (BDG), are established biomarkers for diagnosing invasive fungal infections.

Cell membranes of fungi contain ergosterol as a stabilising agent (as opposed to cholesterol in animal cell membranes). More than 90% of the cell wall of *Aspergillus fumigatus*, however, are formed by different polysaccharides ⁽⁷⁾. These polysaccharides are linked to each other on one hand via covalent bonding, on the other hand via other interactions, to form partly rigid structures, partly loose formations. Relevant polysaccharides are all glucans, mannans, chitin and GM ⁽⁸⁾. Diverse enzymes are responsible for producing these parts of the cell wall. After being produced inside the fungal cell, they are actively transported through the cell membrane to be used, crosslinked, and integrated into the cell wall correctly. Some of the exact mechanisms,

however, need further investigations to be thoroughly understood ⁽⁷⁾. A schematic illustration of *Aspergillus* cell walls is shown in Figure 2.

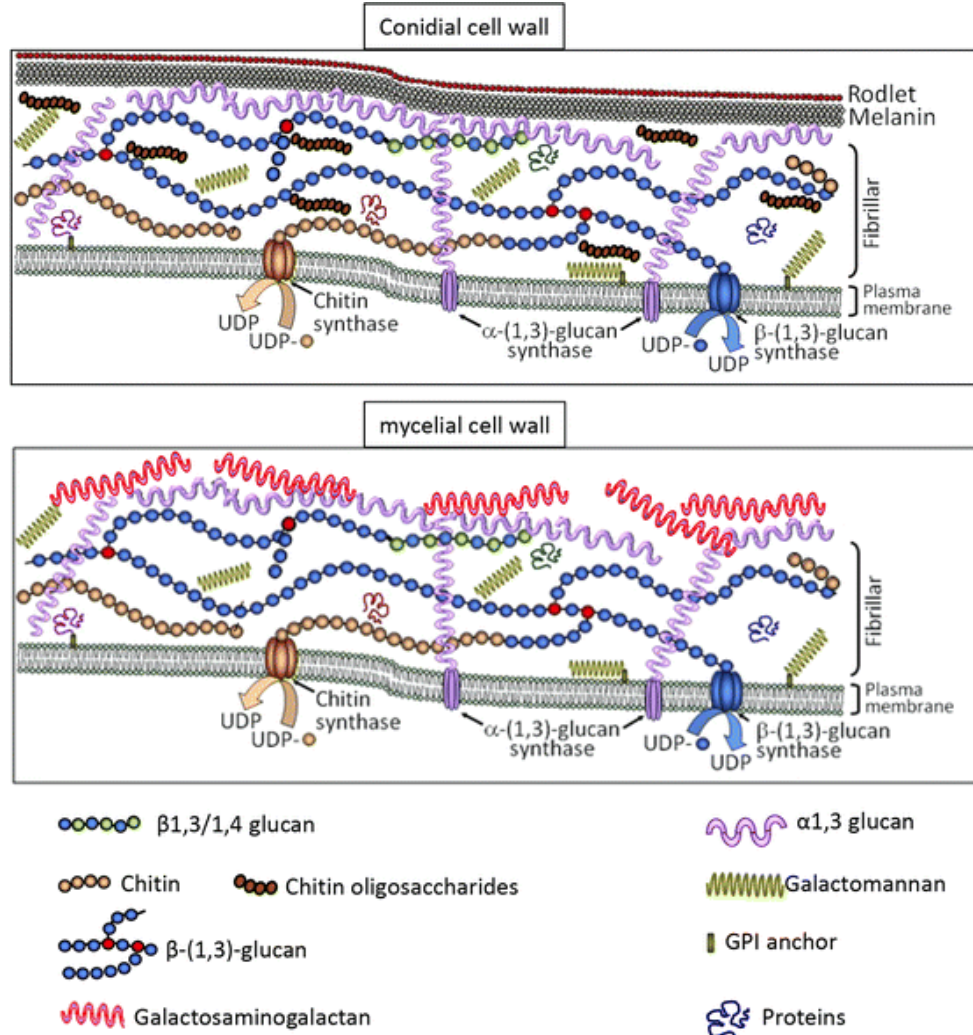


Figure 2. Schematic depiction of the cell wall of the conidia, and the mycelium. Reprinted from ⁽⁹⁾ by permission from Springer Nature. Copyright ©2014 Springer Nature.

There are differences between the composition of conidial cell walls (the dormant form) and hyphae (the germinating form of the fungus), as shown in Figure 3. Although aforementioned polysaccharides can be found in both conidia and the mycelium, they are present in different proportions. In addition to the ordinary cell wall, conidia carry another layer of densely packed proteins, the so-called hydrophobines, and melanin. These layers contribute to different reactions of the human immune systems, especially the phagocytes.

The outer layer of hyphae is built up by galactosaminogalactan, which is absent in conidia (8, 9).

Upon meeting adequate conditions, conidia start swelling) and on their surface, 1,3- α -Glucan is being exposed. Following that, the spores agglutinate and consequently germination commences, leading to synthesis of branched hyphae (9).

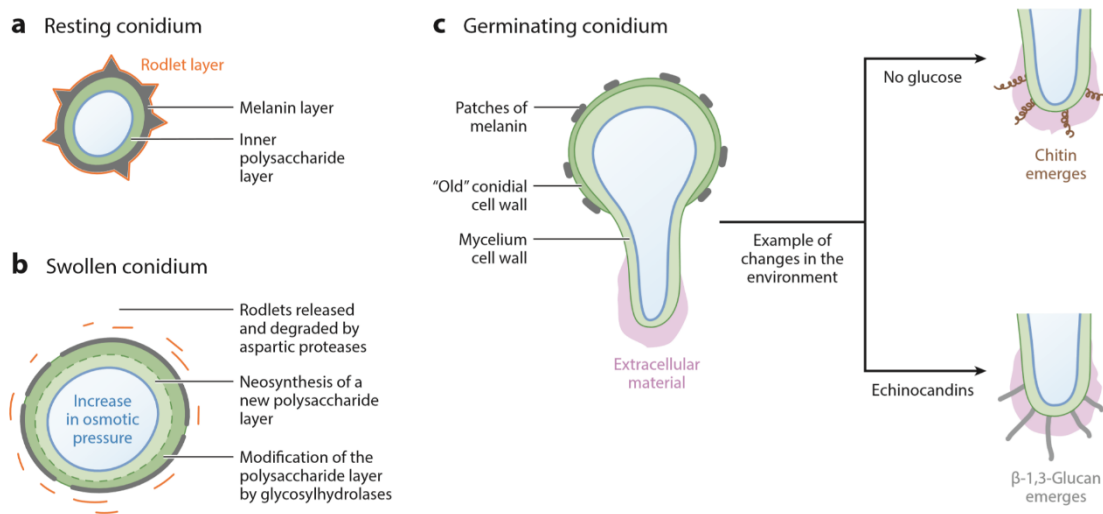


Figure 3. Schematic depiction (a) resting conidium (b) swollen conidium (c) germinating conidium (7). Reprinted with permission from Annual Reviews. Copyright ©Annual Reviews 2017.

6.2 Epidemiology and spectrum of diseases caused by *Aspergillus*

Like mentioned before, *Aspergillus* is a fungus that is ubiquitous worldwide. Just like the fungus itself, its spores are spread widely. Because of their surface morphology, the spores are distributed extraordinarily well in the air (7) and thus inhaled in hundreds daily. For immunocompetent individuals, this constant exposition to spores does not pose a threat: If not instantly transported out of the lung due to the mucociliary clearance and thus being coughed out with mucus, alveolar macrophages as the first line of defence or, alternatively, neutrophils, recognise the spores and phagocytose them (10).

There is a broad spectrum of diseases caused by *Aspergillus*. Depending on the patients' immune status, the spectrum reaches from inadequate local reactions of the immune

system leading to allergies [allergic bronchopulmonary aspergillosis, (ABPA)], over saprophytic forms building cavities [chronic cavitary pulmonary aspergillosis, (CCPA)], to invasive growth [invasive aspergillosis (IA)] and disseminated infections. ⁽¹¹⁾.

The lack of a competent and functional immune system is crucial for developing IA. With a growing number of people suffering from temporary or chronic conditions that impair the immune system over the past years, the number of people at risk for fungal infections has been increasing ⁽¹²⁾. Traditionally, *Candida albicans* is the most prominent cause of fungal diseases. During the past years, though, the relevance of mould fungi grew significantly, most importantly IA and mucormycosis ⁽¹³⁾.

Among the groups with the highest risk of developing IA are people with acute myeloid leukaemia (AML), chronic lymphoproliferative disorders, and patients after receiving allogeneic stem cell transplant ⁽¹⁴⁾. All those patients are considered severely immunosuppressed. However, severe and prolonged neutropenia remains the main underlying risk factor associated with presence of IA ^(15, 16).

Besides persons suffering from haematological malignancies, there are several other groups with an increased risk for developing IA, for instance patients undergoing immunosuppression after solid organ transplantation (SOT). Incidences in this risk group vary between 8.3% (lung transplantation) and 1.2% (liver transplant). Although risk for IA is highest after lung transplantation, mortality proved to be highest after liver transplantation (12-week mortality rate 27.8% vs. 47.1%) ⁽¹⁷⁾. In organ transplant recipients, especially cytomegalovirus (CMV) serological discordance (donor CMV positive and recipient CMV negative), renal insufficiency, surgical re-intervention, and bacterial or viral infection seems to be a risk factor ⁽¹⁷⁾. Further risk factors are displayed in Table 1.

Table 1. Risk factors for IA after SOT ⁽¹⁸⁾

	early IA	late IA (>90 days after SOT)
Lung	Respiratory tract ischemia	Single Lung Transplantation
	Recurrent bacterial infections	Endobronchial prosthesis
	CMV infection	Renal insufficiency
	Previous airway colonisation	Chronic rejection
	Renal failure	

	Renal replacement therapy	
Liver	Re-transplantation	>6g prednisone in the 3 rd month after transplantation
	Renal insufficiency, especially if hemodialysis is required post-transplantation	Hemodialysis post-transplantation
	Liver transplantation due to fulminant liver failure	Renal insufficiency after transplantation
	CMV infection	Post-transplantation leukopenia (<500/mm ³)
	Complicated surgery or re-intervention	
Heart	Isolation of <i>Aspergillus</i> spp. In respiratory tract cultures	Re-admission to the ICU
	Surgical re-intervention	Renal insufficiency post-transplantation
	CMV infection	Concentrations of Tacrolimus >15ng/mL or Cyclosporin >500ng/mL in the 3 rd month after transplantation
	Hemodialysis post-transplantation	>2 episodes of acute rejection
Kidney	Graft rejection	
	Hemodialysis	
	High and prolonged steroid doses	

Patients treated at intensive care units (ICUs) are also considered at risk for developing IFIs. Interestingly, neutropenia is only present in 10-15% of ICU patients. Approximately half of the cases of IA in an ICU affect patients suffering from COPD ^(18, 19). Steroid treatment and COPD proved to increase risk of developing IA significantly ⁽²⁰⁾. Corticosteroids, such as prednisolone, are known to impair both phagocytosis and oxidative functions of polymorphonuclear leukocytes (PMNL) ⁽²¹⁾. Especially high-dose steroid administration diminishes basically all cell lines (myeloid as well as lymphoid) involved in defence against *Aspergillus* ^(22, 23).

6.3 Pathogenesis

Most cases of IA originate from inhalation of spores dispersed in the air. Primary skin infections or infections of the central nervous system (CNS) are alternative ways but are rare.

The ubiquitous dispersion of *Aspergillus* spores is possible because of their hydrophobic surface structure. This hydrophobia is caused by a so called *rodlet layer*. Even in light winds spores are spread easily⁽²⁴⁾. Additionally, the spores have a layer of melanin on their surface, protecting them from UV radiation, making transport in the air possible without damage, and protecting the spores from lysis caused by immune cells⁽³⁾.

After inhalation, mucociliar clearance usually clears the lungs of foreign matter. Because of their small size of about 2-3 μm , the conidia of *Aspergillus fumigatus* can escape mucociliar clearance and can thus reach deeper parts of the respiratory tract easily⁽³⁾.

After escaping mucociliar clearance, the next barrier is formed by alveolar macrophages. Macrophages eliminate dormant conidia in non-oxidative ways. Germinating hyphae, which escape macrophages, are attacked by neutrophil granulocytes and are rendered harmless oxidatively and non-oxidatively⁽²⁵⁾. Macrophages and neutrophil granulocytes recognise pathogens with specific receptors on their surface [pathogen recognising receptors (PRRs)]. TLR-2, TLR-4 and dectin-1 recognise *Aspergillus* and lead to activation of macrophages. In-vitro studies showed that TLR-2 recognises conidia as well as hyphae, whereas TLR-4 recognises only hyphae. Patients after allogeneic stem cell transplantation with TLR-4 polymorphisms are at higher risk for developing IA⁽¹⁰⁾. Phenotypic alterations of the fungus with resulting evasion from recognition by TLR could be a strategy used by *Aspergillus*, contributing to its success as an opportunistic pathogen⁽²⁵⁾.

When reaching a suitable place for growth, swelling conidia present BDG on the surface. In resting conidia, BDG is covered by the *rodlet layer*. Dectin-1 is binding specifically to BDG and plays a major role in the immunological defence for both immunocompetent and immunosuppressed patients. Recognition by dectin-1 leads to activation of macrophages, enhances phagocytosis and causes a strong pro-inflammatory response^(9, 10).

Several factors may contribute to virulence of *Aspergillus*. Just like the *rodlet layer*, the melanin layer on conidia serves as a way of escaping the human immune system. Although melanin itself is immunologically inert, studies with mutants on corresponding genes in the beginning of melanin biosynthesis (PKSB, AYG1, ARP2) showed that conidia of these mutants caused a more intense reaction by dendritic cells, resulting in increased cytokine levels⁽⁹⁾.

Another important factor is immunosuppression caused by *Aspergillus fumigatus*. Like other fungi, *Aspergillus* releases a number of toxins, most prominently gliotoxin. In murine

models it was shown that gliotoxin inhibits macrophage and polymorphonuclear cell function, including phagocytosis and bactericidal activity ⁽²⁶⁾. Gliotoxin also actively inhibits expression of NF- κ B, a transcription factor, and thus preventing transcription of several inflammatory cytokines, hematopoietic growth factors, growth factor receptors, and cell adhesion molecules ⁽²⁶⁾.

6.4 Diagnosis of Invasive Aspergillosis

Early diagnosis of IA is crucial for survival as it represents the cornerstone of early antifungal treatment. A 2001 study ⁽²⁷⁾ demonstrated that prognosis of IA could be improved by reducing time for diagnosis and, thus, initiation of antifungal therapy. Mortality was 90% when adequate therapy for IA was initiated after ≥ 10 days only, whereas it was only 41% when initiated after ≥ 10 days ⁽²⁸⁾. Diagnosis of infectious diseases can often be accomplished by using microbiological cultures. For the diagnosis of IA, however, culture alone is not sufficient for differentiating between colonisation and infection ⁽¹⁶⁾. To establish diagnosis of IA in patients at risk, the EORTC published diagnostic criteria to categorize IA as proven, probable or possible.

6.4.1 Diagnostic criteria

Diagnosis of IA has always been challenging. Researchers and clinicians of the European Organization of Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) started to work on a new way to make diagnosing IA more standardised in 1997. Results of their work have been published in 2002 and 2008.

6.4.1.1 EORTC 2002

Although there are reference standards for diagnosing IA, these involve use of invasive procedures to obtain tissue specimens for culture or histological examination. These procedures are not always feasible, due to underlying diseases of the patients. Clinicians rely on a combination of other, less specific data, such as radiological findings, clinical symptoms and laboratory parameters. To standardise the finding of the diagnosis IA, the EORTC/MSG established diagnostic criteria for clinical researchers. ⁽²⁹⁾.

The committee chose the terms “proven”, “probable” and “possible” to categorise certainty. The basis for these definitions are formed by host factors, clinical manifestations and mycological results ⁽²⁹⁾. The criteria are displayed in Table 2.

Table 2. Diagnostic criteria for invasive aspergillosis according to the 2002 definition from the European Organization of Research and Treatment of Cancer/Mycoses Study Group ⁽²⁹⁾

Proven invasive fungal infections

Deep tissue infections

- Molds

Histopathologic or cytopathologic examination showing hyphae from needle aspiration or biopsy specimen with evidence of associated tissue damage (either microscopically or unequivocally by imaging); or positive culture result for a sample obtained by sterile procedure from normally sterile and clinically or radiologically abnormal site consistent with infection, excluding urine and mucous membranes

Fungemia

- Molds

Blood culture that yields fungi, excluding *Aspergillus* species and *Penicillium* species other than *Penicillium marneffei*, accompanied by temporally related clinical signs and symptoms compatible with relevant organism

Probable invasive fungal infections (host, microbiological, and 1 major (or 2 minor) clinical criteria must be met)

Host factors

- Neutropenia (500 neutrophils/mm³ for 10 days)
- Persistent fever for 96h refractory to appropriate broad-spectrum antibacterial treatment in high risk patients
- Body temperature either 38°C or 36°C and any of the following predisposing conditions: prolonged neutropenia (10 days) in previous 60 days, recent or current use of significant immunosuppressive agents in previous 30 days, proven or

probable invasive fungal infection during previous episode of neutropenia, or coexistence of symptomatic AIDS

- Signs and symptoms indicating graft-versus-host disease, particularly severe (grade 2) or chronic extensive disease
- Prolonged (3 weeks) use of corticosteroids in previous 60 days

Microbiological factors

- Positive result of culture for mold (including *Aspergillus*, or an endemic fungal pathogen from sputum or BALF samples
- Positive result of culture or findings or cytologic/direct microscopic evaluation for mold from sinus aspirate specimen
- Positive findings of cytologic/direct microscopic evaluation for mold or sputum or BALF samples
- Positive result for *Aspergillus* antigen in specimens of BALF, CSF or 2 blood samples
- Positive findings of cytologic or direct microscopic examination for fungal elements in sterile body fluid samples

Clinical factors

Lower respiratory tract infection

- Major

Any of the following new infiltrates on CT imaging: halo sign, air-crescent sign, or cavity within area of consolidation

- Minor

Symptoms of lower respiratory tract infection (cough, chest pain, hemoptysis, dyspnea); physical finding of pleural rub; any new infiltrate not fulfilling major criterion; pleural effusion

Sinonasal infection

- Major

Suggestive radiological evidence of invasive infection in sinuses (i.e., erosion of sinus walls or extension of infection to neighbouring structures, extensive skull base destruction)

- Minor

Upper respiratory symptoms (e.g., nasal discharge, stuffiness); nose ulceration or eschar of nasal mucosa or epistaxis; periorbital swelling; maxillary tenderness; black necrotic lesions or perforation of hard palate

CNS infection

- Major

Radiological evidence suggesting CNS infection (e.g., mastoiditis or other parameningeal foci, extradural empyema, intraparenchymal brain or spinal cord mass lesion)

- Minor

Focal neurological symptoms and signs (including focal seizures, hemiparesis, and cranial nerve palsies); mental changes; meningeal irritation findings; abnormalities in CSF biochemistry and cell count (provided that CSF is negative for other pathogens by culture or microscopy and negative for malignant cells)

Disseminated fungal infection

- Papular or nodular skin lesions without any other explanation; intraocular findings suggestive of hematogenous fungal chorioretinitis or endophthalmitis

Possible invasive fungal infections

- At least 1 host factor criterion; and 1 microbiological or 1 major (or 2 minor) clinical criteria from abnormal site consistent with infection

Abbreviations: AIDS = acquired immunodeficiency syndrome; BALF = bronchoalveolar lavage fluid; CNS = central nervous system; CSF = cerebrospinal fluid; CT = computed tomography

Host factors are meant to represent patients suffering from cancer, whether it may be treated or not, and hematopoietic stem cell transplantation (HSCT) recipients. Mycological evidence is proving if the specimen is obtained from a normally sterile site (such as cerebrospinal fluid), however, if obtained from sites that may be colonised (sputum, BALF, sinus aspirate), evidence is supporting rather than proving. As for clinical manifestations, two categories (major and minor) were created, reflecting differences in importance. Abnormal radiological findings are given a higher rating than unspecific signs such as pleural rub ⁽²⁹⁾.

6.4.1.2 EORTC 2008

Advances in technology and new scientific data led to a process of revision for diagnostic criteria ^(30, 31). The most recent diagnostic criteria, published in 2008 by the EORTC/MSG, represent a revised version of the 2002 criteria. Like the predecessor, diagnosis of invasive fungal diseases is classified into three categories: proven, probable, and possible ⁽³⁰⁾. Possible IA was diminished, as it seemed too permissive ⁽³¹⁾. Minor clinical criteria (see Table 2) were not included in the revised criteria, as specific signs in CT ensure that only patients with sufficient clinical signs are classified ⁽³⁰⁾. While the 2002 criteria required two positive serum GM tests for a patient to be classified as ‘possible’, the revised 2008 criteria only require one. Due to approval of new tests for cell wall components or antigens, microbiological criteria now include both GM and BDG testing in plasma, serum, BALF, or CSF ⁽³⁰⁾. Details of the revised criteria are displayed in Table 4.

Table 3. Diagnostic criteria for invasive aspergillosis according to the 2008 revised definition from the European Organization of Research and Treatment of Cancer/Mycoses Study Group ⁽³⁰⁾

Proven invasive aspergillosis (one of the following points must be met)

Microscopic analysis on sterile material

Histopathologic, cytopathologic, or direct microscopic examination of a specimen obtained by needle aspiration or biopsy in which hyphae or melanized yeast-like forms are seen accompanied by evidence of associated tissue damage

Culture on sterile material

Recovery of *Aspergillus* by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding BALF, a cranial sinus cavity specimen, and urine

Recovery of *Aspergillus* by blood culture

Probable invasive aspergillosis (host, clinical and microbiological criteria must be met)

Host criteria (one of the following must be met)

- Recent history of neutropenia ($<0.5 \times 10^9$ neutrophils/L) for >10 days
- Receipt of an allogeneic stem cell transplant
- Prolonged use of corticosteroids at a mean minimum dose of 0.3mg/kg/day or prednisone equivalent for >3 weeks
- Treatment with other T-cell immunosuppressants such as cyclosporine, TNF- α blockers, specific monoclonal antibodies, or nucleoside analogues during the past 90 days
- Inherited severe immunodeficiency

Clinical criteria (one of the following must be met)

Lower respiratory tract fungal disease

The presence of at least 1 of the following 3 signs on CT scans:

- Dense, well-circumscribed lesion(s) with or without a halo sign
- Air-crescent sign
- Cavity

Tracheobronchitis

- Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopy

Sinunasal infection

Imaging showing sinusitis plus at least 1 of the following 3 signs

- Acute localized pain (including pain radiating to the eye)

- Nasal ulcer with black eschar
- Extension from the paranasal sinus across bony barriers, including into the orbit

Central nervous system infections

1 of the following 2 signs

- Focal lesions on imaging
- Meningeal enhancement on MRI or CT

Mycological criteria (one of the following must be met)

- Direct test (cytology, direct microscopy, or culture) on sputum, BALF, bronchial brush or sinus aspirate indicating presence of fungal elements or culture recovery of *Aspergillus* spp.
- Indirect tests (detection of antigen or cell-wall constituents): Galactomannan antigen detected in plasma, serum, BALF, or CSF

Possible invasive aspergillosis

Presence of host criteria and clinical criteria but absence of mycological criteria

Abbreviations: BALF = bronchoalveolar lavage fluid; CSF = cerebrospinal fluid; CT = computed tomography; MRI = magnetic resonance imaging; TNF- α = tumor necrosis factor alpha

6.4.1.3 Criteria for ICU patients

ICU patients are another group at risk for developing IA ⁽¹⁹⁾ but are not sufficiently represented in the 2008 EORTC/MSG criteria which focus on cancer patients only. According to the EORTC/MSG criteria, IA is diagnosed on a combination of host factors, clinical presentation and microbiological evidence ⁽³⁰⁾. Diagnosing patients treated at ICUs using these criteria, however, is difficult due to various reasons. First, biopsy to establish proven IA is often impossible due to severe underlying conditions. Second, ICU patients

often do not present with classical host factors ^(19, 32) and would thus be considered non classifiable. Risk factors for IA in ICU patients ⁽²²⁾ are displayed in Table 4. Third, typical radiological findings are rare in ICU patients ⁽³²⁾. There are also indicators that biomarkers such as serum GM testing may not be useful in mixed ICU patients ⁽³³⁾. Lastly, Aspergillus recovery in endotracheal samples is common due to mechanical ventilation, immunosuppression, and use of broad-spectrum antibiotics, but could represent colonisation rather than invasive growth ^(20, 32). Because of these challenges, a special algorithm for diagnosing IA in ICU patients (displayed in Table 5) was developed ⁽³⁴⁾.

Nevertheless, diagnosis of IA in ICU patients without classical risk factors still needs to be improved significantly. For this purpose, diagnostic criteria for invasive fungal diseases are currently developed as a standard for ICU patients ⁽³⁵⁾.

Table 4. Risk factors for IA in ICU patients according to Meersseman et al. ⁽²²⁾, by permission of Oxford University Press. Copyright ©2007 Oxford University Press.

High-risk category

- Neutropenia (neutrophil count <500 neutrophils/mm³)
- Haematological malignancy
- Allogeneic 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 for >7 days
- Solid-organ cancer
- HIV infection
- Lung transplantation
- Systemic diseases 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
-

Table 5. Clinical algorithm to discriminate between *Aspergillus* colonization and putative IA in critically ill patients⁽³⁴⁾. Reprinted with permission of the American Thoracic Society. Copyright © 2019 American Thoracic Society.

Proven invasive pulmonary aspergillosis

Idem EORTC/MSG criteria

Putative invasive pulmonary aspergillosis (all four criteria must be met)

1. *Aspergillus*-positive lower respiratory tract specimen culture (= entry criterion)

2. Compatible signs and symptoms (one of the following)

- Fever refractory to at least 3 d of appropriate antibiotic therapy
- Recrudescence fever after a period of defervescence of at least 48 h while still on antibiotics and without other apparent cause
- Pleuritic chest pain
- Pleuritic rub
- Dyspnea
- Hemoptysis
- Worsening respiratory insufficiency in spite of appropriate antibiotic therapy and ventilatory support

3. Abnormal medical imaging by portable chest X-ray or CT scan of the lungs

4. Either 4a or 4b

4a. Host risk factors (one of the following conditions)

- Neutropenia (absolute neutrophil count $<500/\text{mm}^3$) preceding or at the time of ICU admission
- Underlying hematological or oncological malignancy treated with cytotoxic agents

- Glucocorticoid treatment (prednisone equivalent, >20 mg/d)
- Congenital or acquired immunodeficiency

4b. Semiquantitative *Aspergillus*-positive culture of BAL fluid (+ or ++), without bacterial growth together with a positive cytological smear showing branching hyphae

***Aspergillus* respiratory tract colonization**

When ≥ 1 criterion necessary for a diagnosis of putative IPA is not met, the case is classified as *Aspergillus* colonization.

Abbreviations: BAL = bronchoalveolar lavage, CT = computed tomography, EORTC/MSG = European Organization for the Research and Treatment of Cancer/Mycoses Study Group, ICU = intensive care unit, IPA = invasive pulmonary Aspergillosis

6.4.2 *Aspergillus* microscopy and histopathology

Histopathology and direct microscopy showing invasive growth of hyphae of specimens obtained by needle aspiration or biopsy are, along with positive culture of normally sterile material (blood, cerebrospinal fluid etc., excluding urine, bronchoalveolar fluid (BALF) and cranial sinus specimens), proof for invasive aspergillosis ⁽³⁰⁾.

Biopsy is often impractical. Due to underlying diseases that affect coagulation, like haematological malignancies or liver cirrhosis, or generally impaired clinical conditions, like severe pneumonia, invasive procedures may cause complications ^(36, 37). Although proving for IA, histopathology is challenging. Microscopy of *Aspergillus* spp. proved to be difficult, as other fungi tend to mimic *Aspergillus* morphologically, such as *Scedosporium*, *Paecilomyces*, *Scopulariopsis*, and *Fusarium* spp. Additionally, there are morphologic differences in acute and chronic infections. In acute infections, *Aspergillus* hyphae have septate hyphae with dichotomous branching. Hyphal walls are often parallel and show a uniform width. In chronic infections, the hyphae can become larger, distorted, and more tortuous ⁽³⁸⁾. Histological patterns of IA in neutropenic patients versus non-neutropenic patients present differently: in patients with underlying neutropenia, angio-invasion and alveolar haemorrhage are predominating, whereas in patients without neutropenia, tissue invasion, inflammation and necrosis are common, but angio-invasion is uncommon in non-neutropenic patients ⁽³⁹⁾.

Even with experience and thorough microscopy, no histopathological finding can definitely diagnose the pathogen and distinguish between *Aspergillus* spp. and other filamentous fungi and, thus, additional non-cultural based diagnostic procedures are required ⁽¹⁶⁾.

6.4.3 *Aspergillus* culture

Aspergillus culture is an important tool in diagnosis of IA. Although sensitivity in BALF is only about 50% ⁽⁴⁰⁾, culture is of great value as it allows antifungal susceptibility testing, is cheap and simple, and other pathogens such as bacteria can also be identified. Materials for microbiological culture should be submitted in adequate quantities along with brief clinical history, and incubation should start timely to optimize diagnostic yield ⁽¹⁶⁾. Together with clinical symptoms and typical radiological signs, culture allows to diagnose *probable* IA, according to the EORTC/MSG 2008 criteria ⁽³⁰⁾.

Aspergillus fungaemia is a curiosity. Most common underlying disease is acute leukaemia in about 75% ⁽⁴¹⁾. In a study with 36 patients (Kontoyiannis et al., 2000), this condition was found exclusively in patients with haematological malignancies and occurred late in the course of IA ⁽⁴²⁾. From the limited data available it seems that the incidence of *Aspergillus* fungemia is about 2-4% of all cases of fungaemia and more studies are needed to estimate the rate of true aspergillaemia ⁽⁴¹⁾.

6.4.4 Fungal biomarkers for diagnosing IA

4.4.1 *Galactomannan*

Galactomannan (GM) is a polysaccharide produced by fungi and is a component of their cell wall. Growing hyphae and germinating conidia release galactomannan into the bloodstream and various other fluids. Common sources for GM testing are blood/serum and BALF.

A common tool for GM testing is the Platelia *Aspergillus* enzyme immunoassay (EIA), manufactured by Bio-Rad Laboratories (Marnes-la-Coquette, France). False-positive test results may occur. This was, for instance, the case when patients were treated with antibiotics (like Piperacillin/Tazobactam) ⁽⁴³⁾, although more recent studies showed that this was no longer an issue, as it seems that only certain batches were contaminated with

GM. Other false-positive results were also observed in patients receiving parenteral nutrition⁽³³⁾. Patients with other fungal infections, such as *Fusarium* spp., may also test positive for GM⁽⁴⁴⁾. False-negative results are possible when receiving antifungal therapy⁽⁴⁵⁾ and when using dithiothreitol-based mucolytic agents for preparing viscous samples⁽⁴⁶⁾.

Results of GM testing are presented as optical density index (ODI), for which the optical density of a patient's sample is divided by the optical density of a positive control. Recommended cut-off for positivity in serum is ≥ 0.5 ODI, which results in high sensitivity in haematological patients in absence of antifungal prophylaxis. For BALF the optical cut-off is still a matter of debate. However, recent guidelines recommended a cut-off of 0.5 to 1.0 ODI as positivity⁽⁴⁷⁾.

In a 2017 study, GM sensitivity in non-neutropenic patients was shown to be more sensitive in BALF than in serum (75% vs. 37%, cut-off ≥ 0.5 ODI). Thus, BALF testing appears to be more useful than serum testing. Reasons for this are that BALF specimens are taken directly from the site of infection and therefore GM is more abundant, whereas GM in serum may be eliminated by circulating neutrophils⁽⁴⁸⁾. Serum GM testing in neutropenic hosts shows a higher yield than in non-neutropenic hosts; this could be linked to the often higher fungal burden or more angioinvasive growth of *Aspergillus*, leading to higher levels of GM in the bloodstream⁽⁴⁹⁾.

BALF GM testing in immunocompromised patients is more sensitive compared to serum GM testing (85% vs. 23%)⁽⁵⁰⁾. This indicates that BALF GM testing should be performed in both immunocompetent and immunocompromised patients, as sensitivities are comparably high, whereas serum testing is only useful in immunocompromised hosts. Advantage of serum testing is, however, that blood sampling is less invasive than bronchoscopy and can thus be performed more easily.

6.4.4.2 1,3- β -D-glucan (BDG)

As a constituent of fungal cell walls of a variety of fungi, BDG is released into body fluids in association with fungal infections⁽⁴⁷⁾. Presence of BDG in serum signifies fungal invasion but is not specific for *Aspergillus* species, as other fungi, such as *Candida*, *Pneumocystis*, and *Fusarium* produce BDG as well⁽¹⁶⁾. The Fungitell[®] assay is the most common FDA approved BDG assay used (Associates of Cape Cod, Inc., East Falmouth, MA, USA). False-positive results have been reported, for instance when using certain

antibiotics (including some cephalosporins, carbapenems, and ampicillin-sulbactam), and when using cellulose containing materials ^(16, 51). Since *Candida* produces BDG and is part of a normal lung microbiome, BDG levels in BALF are not suitable for diagnosing IA ⁽⁵²⁾.

Cut-off for positivity in serum testing is usually a BDG value of ≥ 80 pg/mL when using the Fungitell assay. BDG testing has been evaluated in case-control studies, with 50-90% sensitivity and 70-100% specificity ⁽⁵³⁾. In neutropenic patients with haematological malignancies, BDG testing shows sensitivity of 60% and specificity of 78% when criterion for positivity is at least one BDG value ≥ 80 pg/mL. When two consecutive positive results occur, sensitivity decreases to 40% while specificity increases to 93%, which is comparable to GM ⁽⁵⁴⁾.

BDG testing plays an important role in guiding antifungal therapy for patients at risk for invasive fungal infections. Overtreatment with antifungals is not only expensive, but also an unnecessary risk for patients to suffer from side effects, drug interactions, and it could even promote resistance. It was shown that negative BDG test results are a safe indicator for discontinuing empirical antifungal therapy in ICU patients, reducing use of antifungal medication ⁽⁵⁵⁾.

6.4.5 Radiology

Since clinical signs and symptoms are not specific for IA, radiological imaging is essential for diagnosis. Not only does it help finding certain signs typical for IA but it also contributes to other diagnostic means, like pointing out sites suitable and promising for BALF, and diagnostic biopsy ⁽¹⁶⁾.

Usually, IA is developing in the lungs first, hence thoracic imaging is most relevant. Whenever IA is suspected, high-resolution CT scan (HRCT) is the method of choice ^(16, 47).

HRCT scans typically show dense, well circumscribed lesions with or without a classical halo sign, air-crescent signs, or cavitary lesions ⁽³⁰⁾. Especially the halo sign, a result of haemorrhage, is regarded to be an early radiological sign of IA in neutropenic patients ⁽¹⁶⁾. The clinical role of the halo sign has been difficult. Greene et al. conducted a study with a large number of individuals with IA included in the Global Comparative Aspergillosis Study. Baseline CT scans of 235 patients were evaluated, 61% of them had halo signs. Patients with halo signs showed significantly better response to antifungal treatment and improved survival ⁽⁵⁶⁾.

CT angiography is another recently studied diagnostic procedure, potentially helpful for discriminating IA and other diseases causing similar radiologic signs in neutropenic patients ⁽⁵⁷⁾, as vessel occlusion, haemorrhage, and angioinvasion seem to be very specific for IA in neutropenic hosts ⁽³⁹⁾.

HRCT may even detect early signs of IA before patients develop symptoms. These baseline CT scans have been found to be predictive for future IA in a study on haematology patients where over a third of pre-symptomatic patients showed radiological signs for IA ⁽⁵⁸⁾.

As mentioned before, CT scans are also useful for other diagnostic strategies. In a 2017 study on immunocompromised patients it was shown that CT guided lung biopsy has high diagnostic accuracy in terms of microscopic examinations. Most common complication is pneumothorax in 19% of cases subsequent of biopsy. Sensitivity, specificity, and positive (PPV) and negative predictive (NPV) values for CT scan were 100, 44, 80, and 100% ⁽⁵⁹⁾, respectively, making it a valid tool for diagnosis of IA.

Other imaging approaches, such as Magnetic Resonance Imaging (MRI), show little profit for diagnosis of IA compared to HRCT or CT angiography. MRI is generally not recommended in baseline diagnosis, as it is time-consuming, technically challenging, and more expensive ⁽¹⁶⁾. While MRI shows a higher sensitivity than CT scans, specificity is poor, and findings in MRI are not as characteristic ⁽⁶⁰⁾. It is, however, the method of choice regarding osseous infections, paranasal sinus infections, and infections of the CNS ^(16,47).

6.5 Treatment of Invasive Aspergillosis

Early initiation of proper antifungal treatment is crucial for survival and good outcome ⁽²⁷⁾. Several classes of antifungal agents are available for treatment of IA: polyenes (Amphotericin B), azoles (Voriconazole, Posaconazole, and Isavuconazole), and echinocandines (Anidulafungin, Caspofungin, and Micafungin).

Choosing the right antifungal agent for patients requires thorough understanding of both the patient and the drug. Side effects can be numerous, underlying diseases may limit the applicability of certain substances, drug interactions need to be kept in mind, and some treatments may require therapeutic drug monitoring.

6.5.1 Polyenes

Amphotericin B (AmB) formulations are the most notable drugs concerning IA in the polyene class. While conventional AmB is known to be nephrotoxic, newer lipid based AmB solutions cause fewer side effects ^(61, 62) and should therefore be preferred. Oral uptake of AmB is poor, so it has to be administered intravenously ⁽¹⁶⁾.

The mechanism of action of AmB has historically been considered due to the formation of ion channels in the fungal cell membrane ⁽¹⁶⁾, but recent studies suggest that fungal cell death is a result of large extramembranous aggregates extracting ergosterol from the cell membrane ⁽⁶³⁾.

AmB formulations are recommended for therapy when azoles are not available or cannot be administered. They may be used in an aerosolized form for prophylaxis, as they are better tolerated than AmB solutions for intravenous administration, and serum levels are negligible ^(16, 64). The recommended dosage for treating IA with liposomal AmB is 3-5mg/kg/day ⁽¹⁶⁾. Higher dosages are not advisable for treatment of IA, as they show no benefit, but higher rates of nephrotoxicity ⁽⁶⁵⁾.

6.5.2. Azoles

Most azoles have broad spectrum activity against *Aspergillus* species and other moulds and fungi. They inhibit ergosterol synthesis by inhibiting fungal cytochrome P450 enzymes that are involved in formation of ergosterol in the fungal cell membrane, leading to inhibition of fungal cell growth and fungal cell death ⁽¹⁴⁾. Azoles are currently the only drugs available for treating IA both orally and intravenously. As plasma levels may vary significantly, therapeutic drug monitoring (TDM) may be required to ensure correct drug levels as well as reduction of adverse effects for some azoles ⁽⁶⁶⁾.

Voriconazole is the most prominent substance among the azole class, as it is considered the first line therapy for IA for a majority of patients ^(16, 67). Metabolization is hepatic, with only 5% of the drug being eliminated renally ⁽¹⁶⁾. This path of metabolization (via CYP2C19 and CYP3A4) suggests that especially patients with underlying liver diseases, such as severe liver cirrhosis, should be closely monitored and dosages may be adjusted ^(68, 69). In a 2002 study among immunocompromised patients (including haematological patients, patients with AIDS, recipients of corticosteroid therapy, and SOT recipients), patients treated with Voriconazole rather than AmB showed a positive outcome of therapy in 52.8% compared to 31.6% in the AmB group ⁽⁷⁰⁾, which is the basis for recommending voriconazole as first line treatment for IA.

Posaconazole is another approved drug of the azole class. It is, however, mostly used and recommended for prophylaxis of IA in recipients of allogeneic HSCT with graft-versus-host disease ⁽¹⁶⁾. As with Voriconazole, prophylaxis (or therapy) with Posaconazole may lead to varying serum drug levels, so TDM is recommended to ensure the achievement of therapeutic levels and avoid toxicities and preventable side effects ^(16, 71). This is especially true for the oral solution, which showed some significant intra-individual plasma levels. As a consequence, a tablet was developed showing a much more predictable uptake after oral ingestion. Thus, TDM for the tablet is not recommended generally but may be of interest in special patient populations (such as patients with severe mucositis) ⁽⁷²⁾.

Isavuconazole is the newest member of the azoles. Its spectrum of activity includes the most common *Candida* species as well as species of *Aspergillus spp.*, and *Mucorales spp.* It requires a loading dose and has a long terminal half-life of 100-130 hours ⁽⁷³⁾. In their SECURE study of 2016, Maertens et al. showed that Isavuconazole is not only non-inferior to Voriconazole for the treatment of IA, but also that, in the ISA arm fewer adverse effects occurred compared to the patients treated with voriconazole⁽⁷⁴⁾. It is licensed as a first-line therapy for IA by the EMA and the FDA. In the current ECIL and ESCMID guidelines Isavuconazole was also recommended as a first line treatment for IA, as was Voriconazole ^(47, 67).

6.5.3 Echinocandins

Echinocandins represent the third main class of antifungal agents used for treating IA. They act by inhibiting synthesis of BDG, weakening osmotic integrity, and interfering with cell growth and cell division ⁽¹⁶⁾. Three drugs of this class are available: Caspofungin, Micafungin, and Anidulafungin. Only preparations for intravenous administration are available.

Contrary to azoles, echinocandins are not recommended as a first-line therapy for IA ⁽¹⁶⁾. Although no lower response rate was monitored in a 2015 study on patients with haematological malignancies and IA treated with Caspofungin rather than Voriconazole, IA mortality was significantly higher ⁽⁷⁵⁾. Additionally, treatment with Voriconazole appears to be economically favourable compared to treatment with echinocandins due to the lower drug cost over the period of treatment ⁽⁷⁶⁾.

6.6 Influence of different diagnostic criteria on epidemiology and outcome of IA

Diagnosis of IA is challenging. Over the past years, the EORTC/MSG has published two guidelines for diagnosing IA (2002 and 2008). The revised 2008 criteria were designed to expand the category of “probable” IA cases, and diminish cases classified as “possible”⁽³⁰⁾. These criteria, however, are not without limitations. ICU patients often present with different signs and symptoms, and lack classic risk factors for developing IA⁽²²⁾. Thus, diagnostic criteria for ICU patients were developed⁽³⁴⁾. To examine the changes in epidemiology and if patients of a certain category would be classified differently with the alterations in diagnostic criteria, comparative work is to be performed.

7. Materials and Methods

7.1 Aim of the study

The primary objective of this study was to compare the three main diagnostic criteria for IA (EORTC/MSG 2002, EORTC/MSG 2008 and ICU criteria ^(29, 30, 34)) in terms of epidemiology and outcome.

The secondary objective was to determine the reasons why patients had been misclassified according to the respective diagnostic criteria.

7.2 Study design characteristics

This study was a retrospective, mono-centric study at the University Hospital of Graz, Austria. From October 2011 to October 2017, clinical data from 617 patients and 899 corresponding BALF samples had been analysed.

7.3 Patients

All samples from patients receiving routine bronchoscopy and BALF for diagnostic work up during the study period at the University Hospital of Graz were included in the study. Decision whether to perform bronchoscopy was up to the treating physician only. Samples were sent to the Department of Internal Medicine, Section of Infectious Diseases and Tropical Medicine for microbiological work-up. Only patients ≥ 18 years of age who were admitted to this hospital were included in this study. If patients were re-admitted to the hospital for further bronchoscopy they were considered for inclusion in this study again.

Inclusion criteria:

- Age ≥ 18 years
- Bronchoscopy with BALF and consecutive microbiological culture

7.4 Data collection and management

Data were collected using MEDOCS (the medical data system used by the KAGes network of hospitals in Styria, Austria). Patients records were thoroughly screened and analysed for

signs and symptoms for IA according to the EORTC/MSG 2002 and 2008 criteria ^(29, 30), as well as the criteria adapted for ICU patients ⁽³⁴⁾. Samples and patients were then sorted into the different diagnostic categories as suggested by the three algorithms. Outcome was examined by using Kaplan-Meier survival curves for the period of one year after classification to the different categories. Survival was measured after 30, 90 and 365 days.

7.5 Statistics

IBM SPSS Statistics 25 (IBM Corporation, Armonk, NY) was used for statistical analysis of collected data. Frequencies of several parameters were calculated. Cumulative survival was calculated using Kaplan-Meier estimators and compared using the Log-rank test for significance.

This study was approved by the local ethics committee (EK 25-221 ex 12/13).

8. Results

A total of 617 patients from whom 899 samples were obtained were included in this study. Main underlying diseases and basic demographic data are displayed in Table 6.

In the study cohort, a total of 102 patients (16.5%) received systemic antifungal treatment.

Table 6. Demographic data and underlying diseases of the study cohort.

		study cohort
No. of patients		617 (100)
Sex		
	male	389 (63)
	female	228 (37)
Age (years)		63 (52-71)
Underlying diseases		
Pulmological		
	ABPA	1 (0.16)
	ARDS	15 (2.43)
	Aspergilloma	2 (0.32)
	Asthma bronchiale	12 (1.94)
	Atelectasis	5 (0.81)
	Bronchiectasis	5 (0.81)
	Bronchitis	6 (0.97)
	COPD	119 (19.29)
	Cystic fibrosis	1 (0.16)
	Hemoptysis	8 (1.30)
	ILD	13 (2.11)
	Lung carcinoma	65 (10.53)
	OSAS	4 (0.65)
	Pleural empyema	3 (0.49)
	Pneumonia	97 (15.72)
	Pulmonary nodules	26 (4.21)
Haematological		
	Agranulocytosis	1 (0.16)
	Acute leukaemia	21 (3.40)
	Aplastic anaemia	1 (0.16)
	Chronic leukaemia	4 (0.65)
	Hodgkin Lymphoma	9 (1.46)
	H SCT	2 (0.32)
	Multiple Myeloma	8 (1.30)

	NHL	22 (3.57)
Malignancies	Breast cancer	7 (1.13)
	Carcinoid	2 (0.32)
	Colorectal Carcinoma	5 (0.81)
	Other solid tumors	39 (6.32)
Internal medicine	Autoimmune disease	47 (7.62)
	Cardiologic diseases	27 (4.38)
	Cushing syndrome	2 (0.32)
	Fever of unknown origin	2 (0.32)
	Immunodeficiency	7 (1.13)
	Liver cirrhosis	15 (2.43)
	Neurological diseases	9 (1.46)
	Peritonitis	3 (0.49)
	Renal insufficiency	9 (1.46)
	Sepsis	10 (1.62)
	SOT	21 (3.40)

Data are given as median (interquartile range) or absolute counts (%)

Abbreviations: ABPA = allergic bronchopulmonary aspergillosis, ARDS = acute respiratory distress syndrome, COPD = chronic obstructive pulmonary disease, HSCT = haematopoietic stem cell transplantation, ILD = interstitial lung disease, NHL = Non-Hodgkin-Lymphoma, OSAS = obstructive sleep apnea syndrome, SOT = solid organ transplantation

8.1 Patients with Proven Invasive Aspergillosis

Two out of 617 (0.3%) patients could be diagnosed with proven Invasive Aspergillosis.

Patient 1 was admitted to the hospital for a coronary artery bypass graft (CABG). Complications occurred during the operation but could be managed and the patient was transferred to the ICU with the sternal wound remaining open for two days. The patient required mechanical ventilation and high doses of catecholamines. Due to high fever the patient received empirical antibiotic therapy; a change of the therapy regimen had to be made due to thrombopenia after receiving Linezolid. On day nine after admission to the ICU, extracorporeal membrane oxygenation (ECMO) was started due to severe acute respiratory distress syndrome (ARDS). GM testing in serum was negative (0.41 ODI) but GM in BALF showed highly positive results (25 ODI). BDG testing in serum, performed on day 13 was highly positive (1460.91 pg/ml) Other parameters such as neutrophils

remained in normal range. Therapy with Voriconazole was started and therapeutic drug monitoring proved that the patient was within required limits (1.5 µg/mL). Culture results in both tracheal secretion and BALF were positive for *Aspergillus fumigatus*. There were no radiological signs for IA in chest X-ray. No computed tomography (CT) was performed. According to the EORTC/MSG 2002 criteria the patient would be diagnosed with possible IA until then. According to the 2008 criteria, the patient would not show sufficient signs for IA, due to the lack of both radiological evidence and host factors. Concerning ICU criteria, the case is classified as *Aspergillus* colonization. The patient died on day 19 after admission to the hospital. Obduction showed multiple pneumonic infiltrates upon macroscopic examination. Microscopy showed hyphae characteristic for *Aspergillus* species in arteries and lung tissue, as well as fresh thrombotic areas. Definite diagnosis of proven IA was established *post mortem*.

Patient 2 was admitted to the Department of Dermatology for treatment of an exanthem, where, upon admission, severe haemolysis was diagnosed. The patient was thus transferred to the Department of Haematology for further diagnostic procedures. Glucose-6-phosphate dehydrogenase deficiency (G6PDD) was assumed. Due to high fever, disseminated intravascular coagulation (DIC), and massive haemolysis, the patient was moved to the ICU on day three. Baseline neutrophils were $1.5 \times 10^3/\mu\text{L}$. Corticosteroid treatment was initiated, together with empirical antibiotic treatment and plasmapheresis in combination with blood transfusions. Examination showed pleural effusions and acidosis. Septic shock was established as a diagnosis. On day five, mechanical ventilation was initiated. The next day brought the final diagnosis of Non-Hodgkin-Lymphoma (NHL). Thus, therapy with Rituximab, Vincristine, and Cyclophosphamide was started. Microbiological tests for fungal infections were negative with the exception of positive enzyme-linked immunoassay (EIA) for *Candida* species. CT scan, performed on day two, showed no specific signs for pneumonia or IA. No antifungal treatment was given. The patient died on day seven. According to all three diagnostic criteria for IA used in this study, the patient did not show sufficient signs for IA. Obduction showed hyphae typical for *Aspergillus* in microscopical examination of the lungs with tissue invasion. Thus, definite diagnosis of proven IA was, again, made *post mortem*.

In both proven IA cases, the three diagnostic criteria could not establish diagnosis *ante mortem*, due to missing host factors in one patient as well as missing radiological findings in both patients. Only obduction showed signs of invasive growth of *Aspergillus*.

8.2 Patients diagnosed with probable or putative IA

When diagnosed according to the EORTC/MSG 2008 criteria, seven (1.1%) patients are categorised with probable IA. Five (71.4%) of these patients were female. Underlying diseases were COPD (n=4), acute leukaemia (n=3), and autoimmune diseases (n=2). None of these patients had other malignancies or SOT as risk factors for IA, while two stayed at the ICU temporally related to date of bronchoscopy. BALF culture results were positive for *Aspergillus* species in four patients. Six (85.7%) received antifungal treatment.

Four (57.1%) of the patients diagnosed with probable IA 2008 were classified as probable IA in the 2002 criteria as well. The other three patients were non-classifiable according to the EORTC/MSG 2002 criteria only due to lack of host factors, and two of them showed positive GM results in BALF.

Demographic data and underlying diseases of patients with probable or putative IA are displayed in Table 7.

Table 7. Demographic data and underlying diseases of patients with probable or putative IA.

	Probable 2008	Probable 2002	Putative ICU
No. of patients	7/617 (1.13)	4/617 (0.65)	4/265 (1.5)
Sex			
male	2/7 (28.57)	1/4 (25)	1/4 (25)
female	5/7 (71.43)	3/4 (75)	3/4 (75)
Age (years)	63 (58-75)	63.5 (59-72.5)	60.5 (33.25-65.25)
Station			
Haematology	2/7 (28.57)	1/4 (25)	0
ICU	2/7 (28.57)	1/4 (25)	4/4 (100)
Pulmonology	3/7 (42.86)	2/4 (50)	0
Underlying disease			
Acute Leukaemia	3/7 (42.86)	2/4 (50)	1/4 (25)
Autoimmune Disease	2/7 (28.57)	1/4 (25)	1/4 (25)
COPD	4/7 (57.14)	2/4 (50)	2/4 (50)
SOT	0	0	1/4 (25)

Data are given as median (interquartile range) or absolute counts (%)

Abbreviations: COPD = chronic obstructive pulmonary disease, ICU = intensive care unit, SOT = solid organ transplantation

Four of 265 ICU patients (1.5%) were diagnosed with putative IA. COPD was present in 2 (50%) of these patients. Two patients were classified as probable IA considering the EORTC/MSG 2008 criteria, the other two were non-classifiable due to lack of host factors.

According to the EORTC/MSG 2002 criteria, four (0.7%) patients could be diagnosed with probable IA; all of them were diagnosed with probable IA using the 2008 criteria as well. Two of these patients were suffering from haematological malignancies (both from acute leukaemia), two from COPD and one had an autoimmune disease. One patient was categorized with putative IA according to the ICU criteria as well. Samples of two patients showed growth of *Aspergillus* species in BALF culture, and one of these patients had highly positive test results in BDG-testing in BALF (3375.9 pg/ml). All four patients were treated with systemic antifungal agents.

8.2.1 Patients with specific CT signs and positive mycological test results without host factors

Additionally, there were seven patients (1.1%) that showed typical signs for IA in CT scans and positive mycological criteria but lacked host factors. These patients are not classified in the 2008 criteria. Their characteristics are shown in Table 8.

Table 8. Non-classifiable patients with CT signs and positive mycological results.

Patient	Underlying disease	Ward	BALF GM	BDG BALF	BDG Serum
1	HIV	ICU Internal	17.0	875	1102.67
2	Liver cirrhosis	Medicine	3.44	0	0
3	Pneumonia	ICU Internal	2.63	0	785.28
4	COPD	Medicine	5.91	0	137.34
5	Liver cirrhosis	ICU	17.78	3912.8	0
6	Acute Leukaemia	Haematology	0.62	519.7	0
7	Pneumonia	ICU	0.84	1871.3	0

Abbreviations: BALF = bronchoalveolar lavage fluid, BDG = 1,3- β -D-glucan, COPD = chronic obstructive pulmonary disease, GM = galactomannan, HIV = human immunodeficiency virus, ICU = intensive care unit. GM testing measured in optical density index (ODI), BDG testing measured in pg/ml.

8.4 Patients diagnosed with possible IA

EORTC/MSG criteria of 2008 diagnosed 13 (2.1%) with possible IA, of which seven (53.9%) were female. One (7.7%) of these patients was staying at the ICU at time of BALF sampling. Risk factors for IA were, most prominently, SOT and haematological malignancies (both found in five patients or 38.5% of patients in this category). No sample showed growth of *Aspergillus* in BALF culture. GM testing in BALF was positive in one sample.

When the EORTC/MSG 2002 criteria were used to diagnose IA, 26 (4.2%) could be categorised as having possible IA. Evenly distributed in terms of gender (50% were male), 13 patients (50%) were staying at the ICU at the time of bronchoscopy. Five (19.2%) were in wards of Pulmonology or Haematology. 15 (57.7%) patients were staying at the ICU temporally related to date of BALF sampling and ten (38.5%) were suffering from haematological malignancies. Two (7.7%) patients' samples showed growth of *Aspergillus* species in BALF culture, and in both patients, GM testing in BALF was positive, so the main reason these patients were not classified as probable IA was missing signs in CT scans. GM in serum, however, remained negative throughout the study case with negative results in BALF culture.

Eight of the 26 possible cases according to the 2002 criteria were classified in the same category applying the 2008 criteria. The others were reduced to non-classifiable due to missing host factors and not showing specific signs in CT.

8.5 Mortality

Both patients with proven IA died (one died two days after diagnosis, the other one eight days after diagnosis). When classified according to the three different criteria, survival rates for 30, 90 and 365 days are displayed in Table 9.

Table 9. Survival rates for 30, 90 and 365 days according to the different criteria and categories. Data are given in percentages. The p value was calculated using the log-rank test.

	30 days	90 days	365 days	p value
Proven	0	0	0	
Probable 2008	83.3	66.7	50	<0.001
Possible 2008	80	80	57.1	
No IA 2008	81.1	77.8	73.1	
Probable 2002	75	75	75	<0.001
Possible 2002	80.8	63.3	41.5	
No IA 2002	81.7	78.8	74	
Putative ICU	75	75	75	0.038
Colonisation ICU	60	60	60	
No IA ICU	60	54.8	53.4	

Kaplan-Meier curves for cumulative survival after one year for the different categories according to the EORTC 2002, EORTC 2008 and ICU criteria are displayed in figures 4-6.

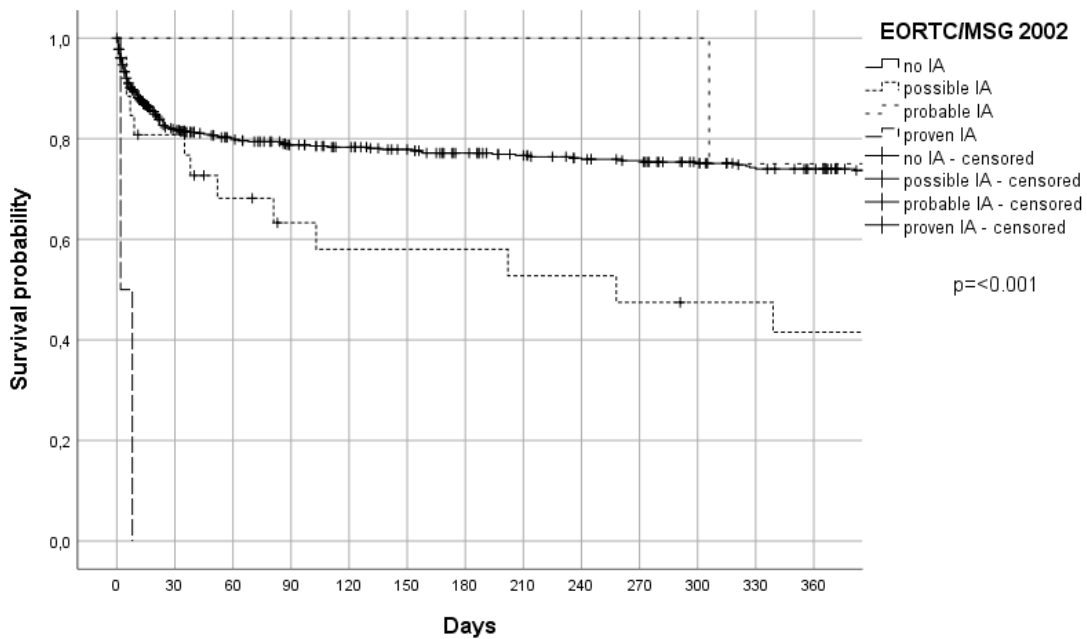


Figure 4. Kaplan-Meier survival curves for the study population categorised according to the EORTC/MSG 2002 criteria⁽²⁹⁾. The p value was calculated using the log-rank test.

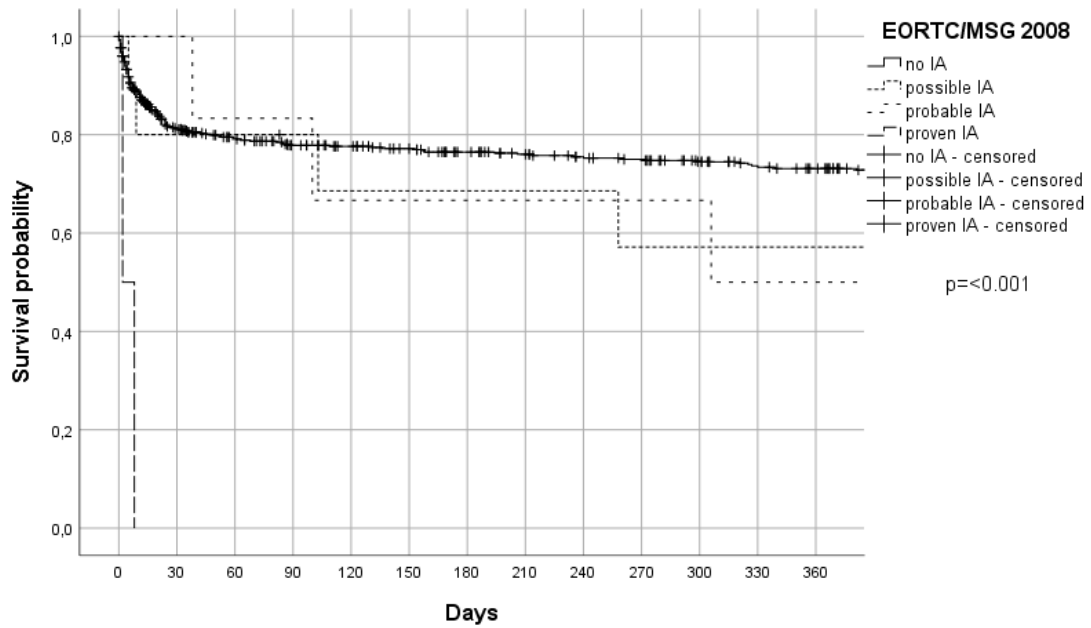


Figure 5. Kaplan-Meier survival curves for the study population categorised according to the EORTC/MSG 2008 criteria⁽³⁰⁾. The p value was calculated using the log-rank test.

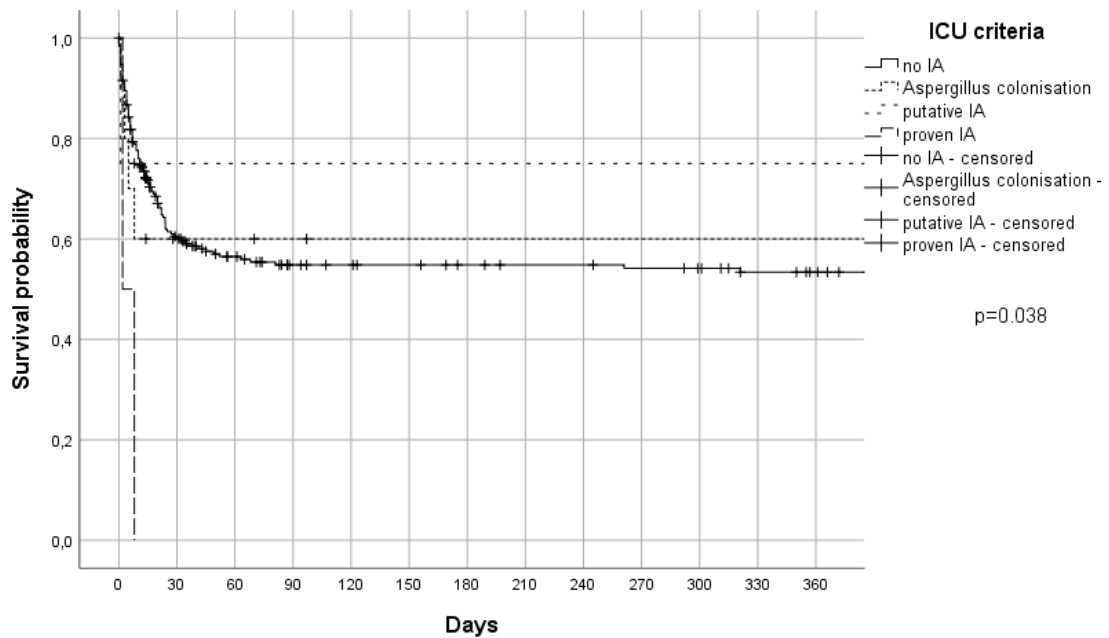


Figure 6. Kaplan-Meier survival curves for the ICU study population categorised according to the ICU criteria⁽³⁴⁾. The p value was calculated using the log-rank test.

9. Discussion

In this study we compared the most commonly used diagnostic criteria for diagnosis of IA and found significant differences among the three diagnostic criteria. Less patients were classified with the 2002 EORTC criteria in contrast to the 2008 EORTC criteria primarily due to lack of host factors. In the ICU setting four patients had putative IA of whom two could not be classified with the EORTC criteria due to missing host criteria.

Two patients in this study were diagnosed with proven IA, both died (two days vs. eight days after diagnosis). None of these two patients were diagnosed with IA according to the criteria mentioned above. This was due to missing host factors and CT signs in one patient and negative microbiological results and CT signs in the other patient. Both patients with proven IA were ICU patients and deceased shortly after bronchoscopy, and diagnosis of IA could only be made *post mortem*, stressing the problem of patients being in clinical conditions unfit to undergo invasive diagnostic procedures. Many cases of IA are missed. This can be for numerous reasons, for instance when clinicians are not trained enough to consider fungal infections, or because potential risk factors like HIV, liver cirrhosis, diabetes mellitus or chronic lymphoid malignancies, or because radiological findings are yet to be recognized, especially in allogeneic stem cell transplant recipients ⁽⁷⁷⁾.

Of seven patients diagnosed with probable IA according to the EORTC/MSG criteria of 2008, which are currently in use, three suffered from acute leukaemia, while four had COPD with ongoing corticosteroid treatment as an underlying disease. Only two of four patients with putative IA according to the ICU criteria were classified as probable IA in the EORTC/MSG criteria. The other two patients lacked classical host factors needed for diagnosis. Lack of a “classical” host factor as requested by the EORTC criteria is a considered an increasing problem, as more patients nowadays are immunosuppressed with new immunosuppressive agents not displayed in the criteria like small molecule kinase inhibitors ⁽⁷⁸⁾ or are suffering from disease causing susceptibility to opportunistic infections (e.g. COPD stage III-IV, advanced liver cirrhosis, severe influenza etc.⁽⁷⁹⁻⁸¹⁾). Those patients are currently not adequately represented in the most recent IA definition. All four patients diagnosed with probable IA according to the 2002 criteria were classified in the same category when applying the 2008 criteria. Number of possible IA cases was reduced from 26 (EORTC/MSG 2002) to 13 (EORTC/MSG 2008), this was due to missing host

factors and lack of specific signs for IA in CT scans. They were consequently non-classifiable.

In this study, patients with probable IA nearly doubled when comparing the EORTC/MSG 2002 and 2008 criteria (0.7% vs. 1.1%), emphasising that the intention of expanding the category of probable IA, as intended by the EORTC/MSG, proved useful. A broader use of microbiological testing (including GM, BDG, and polymerase chain reaction) improved diagnostic possibilities, leading to quicker and better diagnosis. All patients in this category showed classical risk factors for IA, notably haematological malignancies and COPD. Concerning patients with possible IA, the number of cases were cut down drastically (4.2% vs. 2.1%). A single-center retrospective analysis by Tsitsikas et al. in 2012 showed similar results⁽⁸²⁾.

When using the 2008 criteria, patients with probable IA had a survival probability of 50% after 365 days of follow-up, compared to a substantially higher probability of 75% according to the 2002 criteria. Although the number of cases is very low, together with the reduction of possible IA cases this may indicate that the 2002 criteria were indeed too generous when sorting patients into the category of possible IA. Nevertheless, the two patients with proven IA in this study were missed by the EORTC criteria and could only be diagnosed *post mortem*. This was due to missing host factors in one patient and lack of specific findings in CT scans.

Seven (1.1%) patients showed specific signs for IA in CT scans and had positive mycological test results, like GM in BALF, but no host factors. Four of them were ICU patients. According to the EORTC/MSG criteria, they are considered non-classifiable. Two of these patients had liver cirrhosis as an underlying disease, a risk factor for IA just recently proposed⁽⁸⁰⁾, others had HIV or COPD. This is again stressing the potential role of broadening the range of host factors in diagnostic criteria that may be published in the future.

The ICU criteria were published by Blot et al.⁽³⁴⁾ to solve another problem in the diagnosis of IA: ICU patients often lack classical host factors and specific CT signs needed for being classified in the EORTC/MSG criteria and discriminating between colonisation and infection^(19, 22). In ICUs, incidences of IA up to 5.8% were reported⁽²²⁾. In this study, four of 265 (1.5%) ICU patients were diagnosed with putative IA, but only two of them were classified as probable IA using the 2008 criteria. This was due to a lack of host factors.

Two of these patients had COPD, which is a risk factor for ICU patients. More studies are needed to establish a more secure way of diagnosing IA in critically ill patients, as they are often not immunocompromised, and diagnostic procedures vary in their performance in these patients. For instance could a delay in starting empirical treatment for fungal infections in favour of further diagnostics be better for diagnosis and outcome in some cases ⁽³⁵⁾.

Diagnosing IA remains difficult. In this study, 102 of 617 patients (16.5%) received antifungal treatment for either having suspected IA or having high risk of developing IA. Together with the rather low number of probable and proven IA cases found in this study, this may be an indicator of shortcomings in the diagnostic criteria currently in use for clinical research. There are developments in diagnostic biomarkers, such as lateral flow devices ⁽⁸³⁾ or Triacetylfusarinine C in urine ⁽⁸⁴⁾, that will eventually show ways to diminish these gaps.

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