Dissertation

Invasive Mould Infections in Patients with Haematological Malignancies: Novel Diagnostic Approaches

submitted by

Dr. med. univ.

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz“.

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Related Papers

Parts of this thesis have been published in these papers with the permission of all co-authors. Permission to reproduce and adapt figures and tables has been obtained from the respective copyright holders.

Eigl S., Hoenigl M., Spiess B., Heldt, S., Prattes J., Neumeister P., Woelfler A., Rabensteiner J., Prueller F., Krause, R., Reinwald M., Flick H., Buchheidt D., Boch T.; **Galactomannan testing and Aspergillus PCR in same-day bronchoalveolar lavage and blood samples for diagnosis of invasive aspergillosis.** Med Mycol. 2017 Jul 1;55(5):528-534. doi: 10.1093/mmy/myw102.
License provided by the International Society for Human and Animal Mycology (ISHAM).

Eigl S., Prattes J., Reinwald M., Thornton C. R., Reischies F., Spiess B., Neumeister P., Zollner-Schwetz I., Raggam R.B., Flick H., Buchheidt D., Krause R., Hoenigl, M. **Influence of mould-active antifungal treatment on the performance of the Aspergillus-specific bronchoalveolar lavage fluid lateral-flow device test.** Int J Antimicrob Agents. 2015 Oct;46(4):401-5. doi: 10.1016/j.ijantimicag.2015.05.017. Epub 2015 Jun 30**.**

Eigl S., Prattes J., Lackner M., Willinger B., Spiess B., Reinwald M., Selitsch B., Meilinger M., Neumeister P., Reischies F., Wölfler A., Raggam R.B., Flick H., Eschertzhuber S., Krause R., Buchheidt D., Thornton C.R., Lass-Flörl C., Hoenigl M.; **Multicenter evaluation of a lateral-flow device test for diagnosing invasive pulmonary aspergillosis in ICU patients.** Crit Care. 2015 Apr 17;19:178. doi: 10.1186/s13054-015-0905-x.

Parts of the results were presented at the 55th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICCAC), 17.-21. September 2015, San Diego, CA, USA.

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Table of contents

1 Abbreviations

A

ABPA = Allergic Bronchopulmonary Aspergillosis

AF = Antifungal

AIDS = Acquired Immunodeficiency Syndrome

ALL = Acute Lymphocytic Leukaemia

AML = Acute Myelogenous Leukaemia

AmB = Amphotericin B

B

BALF: Bronchoalveolar Lavage Fluid

BDG = Beta-D-Glucan = 1,3-β-D-Glucan

C

CCPA = Chronic Cavitary Pulmonary Aspergillosis

CGD = Chronic Granulomatous Disease

CLL = Chronic Lymphocytic Leukaemia

CPA = Chronic Pulmonary Aspergillosis

CT = Computer Tomography

CTPA = Computer Tomography Pulmonary Angiography

D

DOR = Diagnostic Odds Ratio

E

EBMT = European Society for Blood and Marrow Transplantation

ECIL = European Conference on Infections in Leukaemia

E.g. = "exempli gratia" = for example

ELISA = Enzyme-Linked Immunosorbent Assay

ELN = European Leukaemia Net

EORTC = European Organization for Research and Treatment of Cancer

Et al = et alii/ et aliae

F

FDA = U.S. Food and Drug Administration

G

GM = Galactoamannan

GvHD = Graft versus Host Disease

H

HSCT = Hematopoietic Stem Cell Transplantation

I

IA = Invasive Aspergillosis

ICSH = International Immunocompromised Host Society

ICU = Intensive Care Unit

IDSA = Infectious Diseases Society of America

IFI = Invasive Fungal Infections

IMI = Invasive Mould Infections

IOM = Institute of Medicine

IPA = Invasive Pulmonary Aspergillosis

L

LFD = Lateral Flow Device Test

M

MBL = Mannose-Binding Lectin

MDS = Myelodysplastic Syndrome

MM = Multiple Myeloma

MSG = Mycoses Study Group

N

NHL = Non-Hodgkin Lymphoma

NPV = Negative Predictive Value

O

ODI = Optical Density Index

P

PPV = Positive Predictive Value

S

SOT= Solid Organ Transplantation

spp. = species pluralis

W

WHO = World Health Organization

2 List of figures

3 List of tables

4 Abstract

4.1 Introduction

The inability to make an early and convincing diagnosis still remains a central problem in the diagnosis of invasive aspergillosis (IA). In the last years new diagnostic tests were developed. Galactomannan testing (GM) and *Aspergillus* PCR have both become essential for diagnosis of IA in patients with haematological malignancies. Whether or not PCR and GM need to be performed in bronchoalveolar lavage fluid (BALF), a sample from the direct site of infection, or testing of blood samples is sufficient, remains unknown.

4.2 Methods

We evaluated the diagnostic performance of GM ELISA, and *Aspergillus* specific PCR by using BALF samples and blood specimens obtained at the same day. In case of suspected IA and when a diagnostic bronchoscopy was performed in clinical routine, BALF aliquots were reserved for study purposes and additionally whole blood sample were collected within 24 hours of bronchoscopy. GM testing was performed at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Austria. BALF aliquots together with whole blood samples, were shipped overnight to the scientific laboratory in Mannheim, Germany for BALF *Aspergillus* specific and panfungal PCR testing. Additionally, we expand our cohort by including cases from Mannheim, which were enrolled retrospectively.

4.3 Results

A total of 53 immunosuppressed patients were included in the final analysis, with 16 probable/proven IA and 37 with no evidence of IA according to the revised EORTC/MSG criteria. 38 patients with haematological malignancies were prospectively enrolled at the Medical University of Graz, Austria, 15 patients with mixed underlying diseases at the Mannheim University Hospital, Germany. Patients with possible IA were excluded from this analysis. A total of 34/53 (64%) of all patients and12/16(75%) of patients with probable/proven IA received antifungal treatment at the time of the BALF recovery. Sensitivities of *Aspergillus* PCR and GM were 44% and 38% in BALF, and 0% and 31% in blood, respectively. BALF PCR results demonstrated to be superior (sensitivity 44%,

specificity 100%) compared to blood PCR. Combination of BALF *Aspergillus* PCR, BALF GM (>1.0 ODI), BALF-culture and/or serum GM (>0.5ODI) resulted in the highest sensitivity (75%) for probable/proven IA cases.

4.4 Conclusion

In conclusion sensitivity was low in every evaluated diagnostic test when interpreted on their own (low in BALF and even lower in blood). In our patient cohort diagnostic test combination resulted in a significant higher diagnostic performance and could improve diagnosis and management of invasive aspergillosis. In addition, combination of PCR and GM testing and culture from BALF resulted in higher sensitivities, without markedly impacting specificity.

5 Zusammenfassung

5.1 Einleitung

Die frühe Diagnosestellung von invasiven Aspergillosen (IA) bei immunsupprimierten Patienten ist nach wie vor ein zentrales Problem. In den letzten Jahren wurden die diagnostische Tests ständig erweitert und kontinuierlich verbessert. Der Galactomannan-Test (GM) und die *Aspergillus*-PCR sind für die Diagnosestellung mittlerweile unverzichtbar geworden. Ob der Nachweis von Pilz-DNA oder von Zellwandkomponenten in bronchoalveolarer Spülflüssigkeit (BALF) als Probe vom direkten Infektionsort oder in den einfacher verfügbaren Blutproben durchgeführt werden soll, wird weiterhin in Expertenkreisen diskutiert.

5.2 Methodik

Wir untersuchten die diagnostische Leistung des GM-ELISA und der *Aspergillus*-spezifischen PCR unter Verwendung von BALF- und Blutproben, die am selben Tag entnommen wurden. Bei Verdacht auf invasive Aspergillose und folglich durchgeführter Bronchoskopie wurden BALF-Aliquote für die Studienzwecke reserviert und zusätzlich wurden maximal 24 Stunden nach BALF-Gewinnung, häufig direkt nach oder vor der Intervention, Blutproben abgenommen. GM Bestimmung in den BALF- und Blutproben wurde am Klinischen Institut für Medizinische und Chemische Labordiagnostik der Medizinischen Universität Graz, Österreich, durchgeführt. BALF-Aliquote wurden zusammen mit den Blutproben über Nacht an das wissenschaftliche Labor in Mannheim zur Durchführung der *Aspergillus*-spezifischen und panfungalen PCR-Tests versandt. Wir erweiterten unsere Kohorte schließlich noch um Fälle aus Mannheim, die retrospektiv eingeschlossen wurden, um eine ausreichende Anzahl von Fällen zur Berechnung und damit eine höhere Aussagekraft zu erzielen.

5.3 Resultate

Insgesamt 53 immunsupprimierte Patienten wurden in der finalen Analyse inkludiert. Gemäß der überarbeiteten EORTC/MSG-Kriterien hatten 16 eine wahrscheinliche/nachgewiesene IA und 37 ohne keinen Hinweis auf eine invasive Schimmelpilzerkrankung. 38 Patienten hatten eine hämatologische Grunderkrankung und

wurden prospektiv an der Medizinischen Universität Graz, Österreich, eingeschlossen, 15 Patienten mit gemischter Grunderkrankung am Universitätsklinikum Mannheim, Deutschland. Patienten mit möglicher IA wurden von der Endanalyse ausgeschlossen. Insgesamt 34/53 (64%) aller Patienten und 12/16 (75%) der Patienten mit wahrscheinlicher/nachgewiesener IA erhielten zum Zeitpunkt der BALF-Gewinnung eine antimykotische Therapie. Die Sensitivität von *Aspergillus* PCR und GM betrugen 44% bzw. 38% in BALF und 0% bzw. 31% in Blut. Die BALF-PCR-Ergebnisse erwiesen sich unter allen diagnostischen Tests und Proben als überlegen (Sensitivität 44%, Spezifität 100%). Kein Patient mit IA wäre allein durch die PCR aus verifiziert worden. Die Kombination von *Aspergillus*-PCR und GM (> 1,0) in BALF, BALF-Kultur und GM (> 0,5ODI) im Serum (Spezifität 95%) ergab die höchste Sensitivität (75%).

5.4 Konklusion

Zusammenfassend war die Sensitivität in allen bewerteten diagnostischen Tests bei alleiniger Interpretation niedrig (niedrig in BALF und sogar noch niedriger in Blut). In unserer Patientenkohorte führte die Testkombination zu einer signifikant höheren diagnostischen Leistung und konnten die Diagnose und das Management invasiver Schimmelpilzinfektionen verbessern. Darüber hinaus führte die Kombination von PCR, GM-Test und BALF-Kultur zu höheren Sensitivitäten, ohne die Spezifität merklich zu beeinflussen.

6 Introduction

6.1 Background

6.1.1 The kingdom of fungi

In the past fungi were, in comparison to bacterial and viral pathogens, underappreciated. This rapidly changed in the last decades due to the fact that fungal diseases reduced the tree population diversity, changed forest ecosystems, caused crop loss and were responsible for extinctions in wildlife, as well as for death and disability in humans (1-4).

Previously taxonomically counted as a member of the plant kingdom, today fungi represent the third biggest of the six eukaryotic kingdoms and have more in common with animals than plants (5). It is supposed that the actual number of fungal species is 2.2 to 3.8 million; only 120.000 are currently known and hence just 8% are identified and described so far (6). But this range is probably underestimated; there also exists estimates up to 5.1 million fungal species (7, 8). Not much is known about the fungal biodiversity and biogeography, which is why scientists also refer to them as “The Hidden Kingdom”.

Fungi were simply classified into filamentous moulds (e.g. *Aspergillus spp.*, *Mucor spp.*), dimorphic fungi (e.g. *Histoplasma spp.*, *Blastomyces spp.*), and yeasts (e.g. *Candida spp.*, *Cryptococcus spp.*). However, the fungal organism is far from simple and is the most diverse organism on the planet. They are ubiquitous and exist in an enormous variety of sizes, shapes and colours, as single-celled organisms or complex communities (7, 9). This variety is displayed in figure 1. Moreover, fungi are strongly adaptable under extreme conditions like e.g. high salt concentrations, extremely high and low temperatures or acidic and basic environments. These stressful habitats are hostile to most eukaryotic organisms. Gostinčar et al. suggest that the precondition for this ability for evolutionary adaptation of the fungi are processes like asexuality, synthesis of melanin-like pigments and a flexible morphology (10). In the same year Heitmann in turn published that fungi have cryptic sexual cycles, including unisexual or parasexual reproduction. This covert sexual reproduction of the eukaryotes allows the genetic diversity to delete destructive mutations and produce better-fitting progeny (11). So, multiple strategies for reproduction enable the fungal organism to become niche and host-adapted and leads to the evolution of extremophiles and furthermore to the formation of novel fungal pathogens.

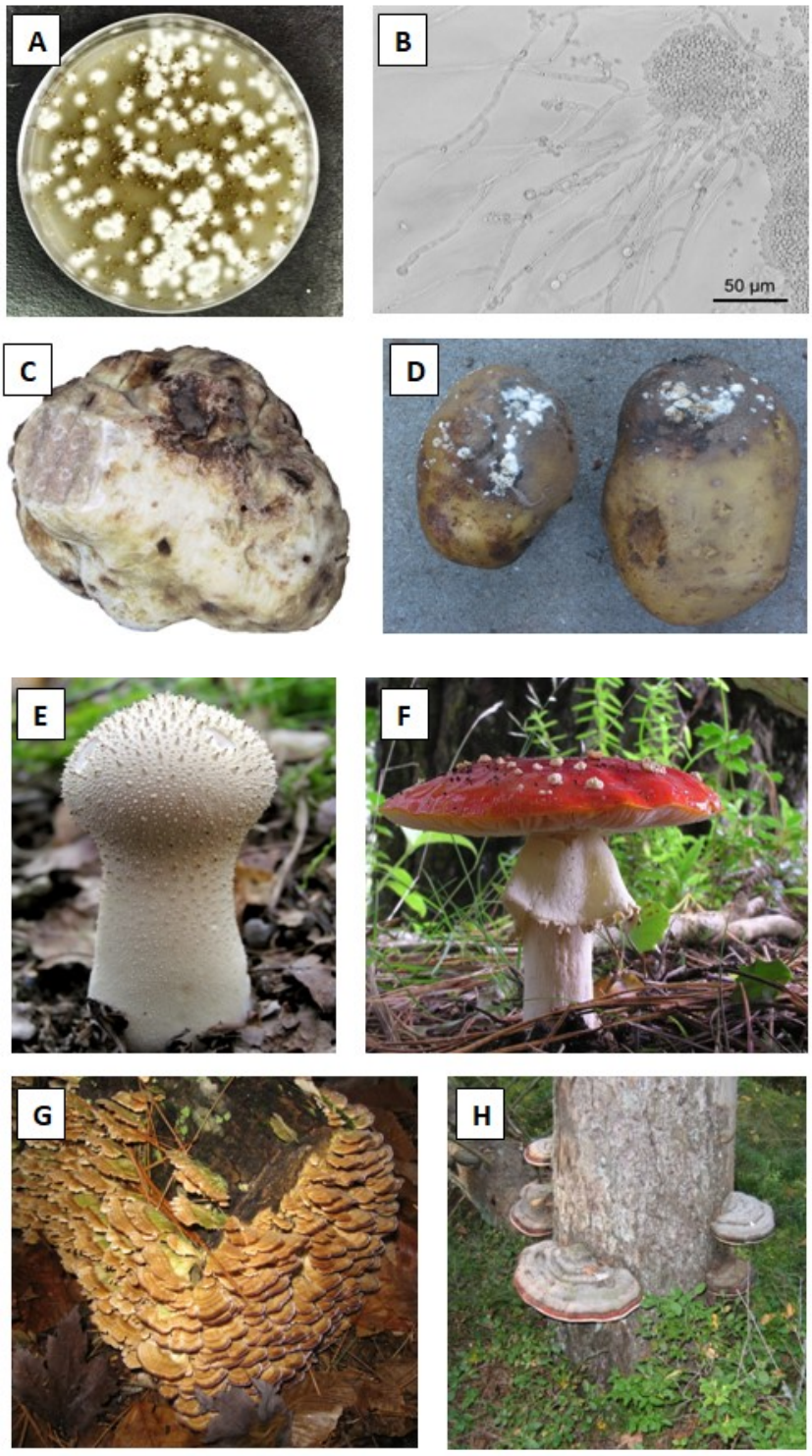


Figure 1: Diversity of fungal morphology: (A) Cryptococcus gattii isolation, by Djspring, distributed by Creative Commons Attribution-Share Alike 3.0 license. No changes were made; (B) Microscopic image Candida albicans, by Y tambe, distributed by GNU Free Documentation License. No changes were made; (C) White truffle, by Matthias Kabel, distributed by GNU Free Documentation License. No changes were made; (D) Potato late blight - Phytophthora infestans, by Rasbak, distributed by GNU Free Documentation License. No changes were made; (E) Lycoperdon perlatum, by Daniel Ullrich distributed by GNU Free Documentation License. No changes were made; (F) Amanita muscaria, by Tony Wills, distributed by Creative Commons Attribution-Share Alike 3.0 license. No changes were made; (G) Polypores (mushrooms in the genus Trichaptum; (H) Fungus Polyporacea, by Ronja Addams-Morin, distributed by GNU Free Documentation License. No changes were made.

6.1.2 Fungi - Good or bad?

Fungal organisms are essential for life. They are able to degrade complex organic matter and recycle essential nutrients back to the environment, live in symbiosis with plenty of animals and plants and are a direct food source as well (12-14). Not to mention one of the biggest achievements in medical healthcare: fungal derived antibiotics. Fungi were in addition used for the fabrication of immunosuppressants, statins and anti-cancer medication (15, 16). One of the best-known representatives of the fungal kingdom used to produce medication and food is *Penicillium spp.* (figure 2).

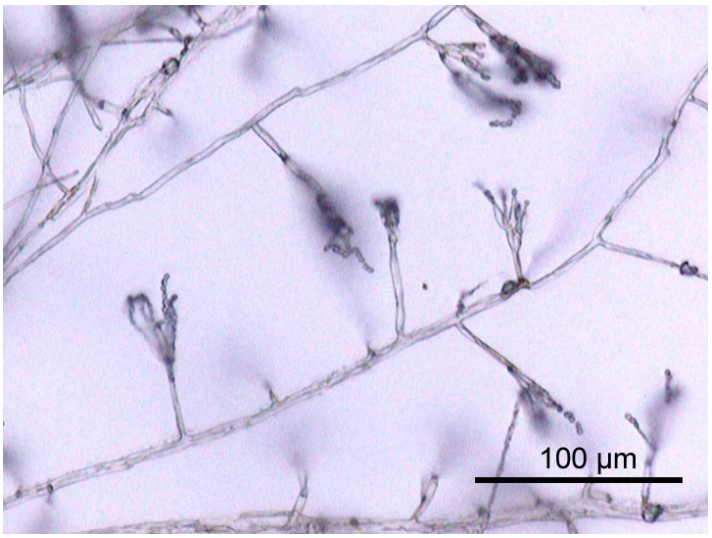
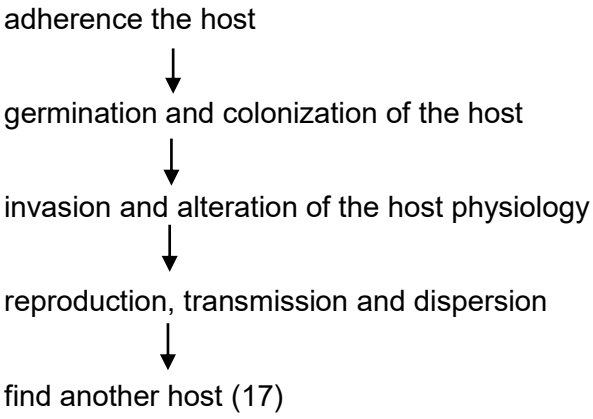


Figure 2: *Penicillium spp.* under microscope, by Y_tambe, distributed by GNU Free Documentation License. No changes were made.

Concurrently, invasive fungal infections are a life-threatening disease. At the beginning of the 20th century only one, previously unknown pathogenic fungus was responsible for the eradication of the whole chestnut tree population in the east of the United States (2). *Phytophthora infestans*, the causative agent of potato late blight (figure 1, D), was the major culprit of the hunger crises and the death of millions of people in Ireland in the 19th century. *Cryptococcus gattii* induced an epidemic of animal and human infections and deaths in Canada in 1999 (3, 4). In 2010 the Forum on Microbial Threats of the Institute of Medicine (IOM) arranged a workshop about fungal diseases with the title “Emerging Threat to Human, Animal and Plant Health”. “Fungi are the only group of organisms that have been convincingly shown to cause extinction” were the words of Arturo Casadevall in

2010, a professor of molecular microbiology and immunology and infectious diseases and an internationally recognized expert in infectious disease research.

To be a pathogen an organism must be sophisticated and able to evade immune response and accordingly cause disease in a host. Fungal pathogens share many of their biological processes with humans. These sequential steps were published for the fungal to be pathogenic:



Each step often involves common mechanisms. Some fungi are able to infect humans, plants and animals (trans-kingdom pathogens); others are only pathogenic to animals and humans, but non-pathogenic to plants (18, 19). Outside of the host, fungi also have different approaches for survival before affecting the next host – many do not even need a living individual to survive (20). Diseases are categorized as “emerging” if their incidence or virulence has recently increased or if they begin to infect a novel host or population (WHO, 2010). Thirteen factors were detected and shown as significant for the emergence of infectious diseases in 2003 in an Institute of Medicine report (*Microbial Threats to Health Emergence, Detection and Response*) (21):

- Microbial adaptation and change
- Human susceptibility to infection
- Climate and weather
- Changing ecosystems
- Human demographics and behaviour
- Economic development and land use
- International travel and commerce
- Technology and industry
- Breakdown of public health measures
- Poverty and social inequality

- War and famine
- Lack of political will
- Intent to harm

Over the last 200 years, easier, cheaper and faster transport technology led to an enormous increase in human mobility (1.000-fold in Western countries) and the transfer of infectious diseases worldwide (22, 23). Figure 3 shows only a part of the global aviation network. Travel and trade were responsible for the majority of emerging fungi diseases in crop plants, because they were spreading via global transportation. As a result fungi diseases raised economic loss and had consequences in human wellbeing and biodiversity (24). Since the first outbreak of *Cryptococcus gatii* as an emerging fungal infection in humans on Vancouver Island, Canada, in 1999, the fungus, which was previously restricted to tropical and subtropical regions, spread from Canada to the northwest of the United States (25).

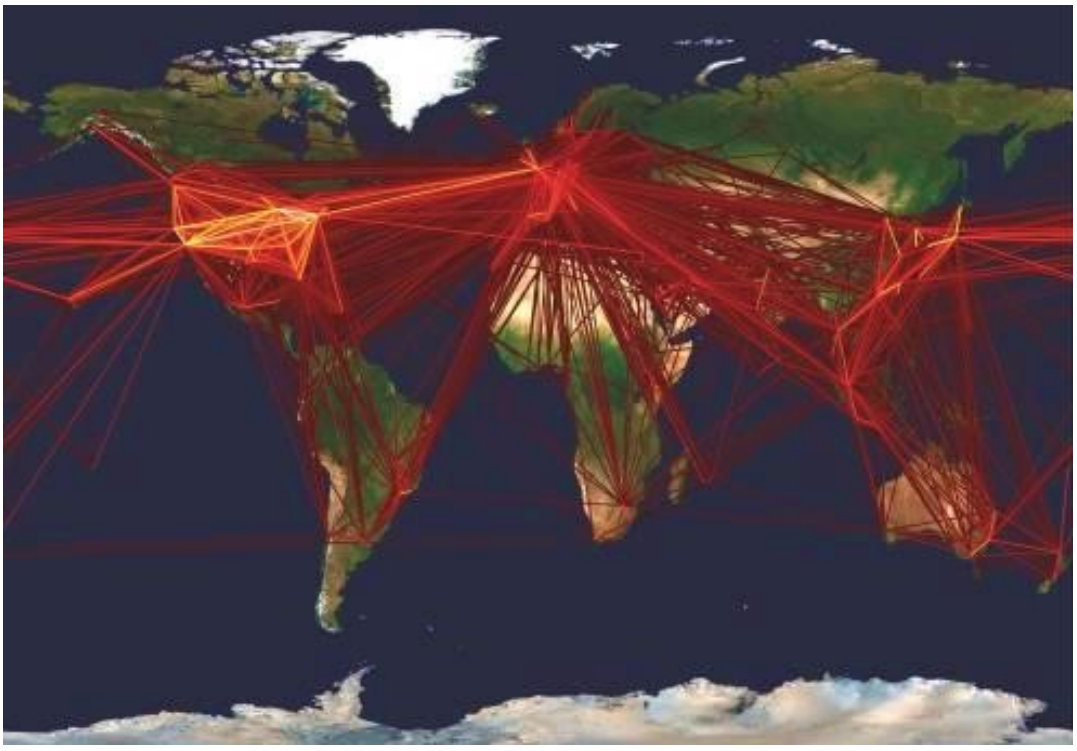


Figure 3: Global aviation network. Each line represents a direct connection between the 500 largest international airports in >100 different countries. From: Hufnagel L, Brockmann D, Geisel T. Forecast and control of epidemics in a globalized world. Proc Natl Acad Sci U S A. 2004;101(42):15124-9. Copyright (2004) National Academy of Sciences, U.S.A. No changes were made (23).

6.2 Invasive fungal infections

Invasive fungal diseases are infections of the internal organs or blood stream up to sepsis caused by pathogen fungi. Until now around 300 fungal species are known that can be pathogenic to humans, though only less than 12 fungal species are able to provoke a life-threatening invasive mycoses (26). Hence being pathogenic is not characteristic for most fungi.

The fungal pathogen usually enters the human organism in the form of asexual spores via respired air or via the digestive system; hence the lung is the most common organ for fungal infections. Almost exclusively affected are patients with a restricted immune response like in patients with haematological malignancies or solid-organ transplant recipients (27, 28). Since the 1950s the increasing number of immunosuppressed patients provoke a growing incidence of fungal infections worldwide, and the clinical relevance is higher than ever before (29, 30). Not only the underlying disease but also the aggressiveness of the appropriate medical therapy induces the immunocompromise and promotes the risk for invasive fungal infections. At highest risk are patients with prolonged neutropenia after stem cell transplantation or after the administration of chemotherapy.

In this regard, from the 13 factors mentioned above, medical advances and new therapeutic options are the most fundamental factors leading to the emergence of fungal infections in humans in the 20th century. “Non-pathogens” become “pathogens” and fungal organisms become more important to human health than ever before. Aya Homei summed it up in “Candida: A Disease of Antibiotics” and Michael Worboys in “Aspergillosis: A Disease of Modern Technology” (31). In the United States the rate of sepsis caused by invasive fungal infections increased more than 200 percent between 1979 and 2000, and mucormycosis-associated hospitalizations doubled between 2000 and 2013 (32, 33). The increasing incidence of fungal diseases connected to the improvements in medical history is shown in figure 4.

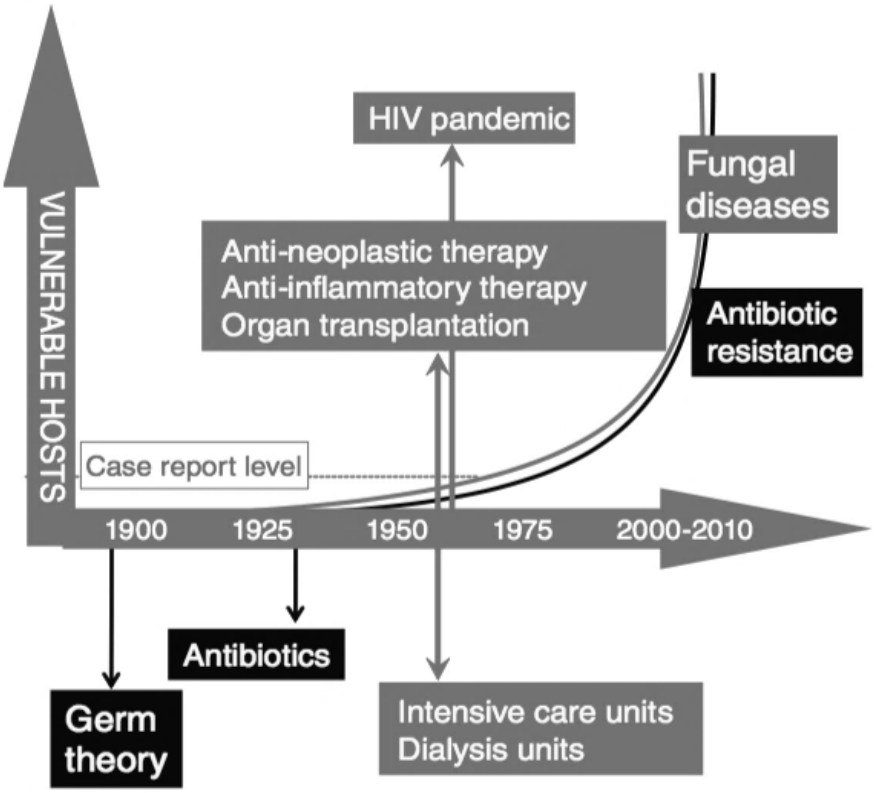


Figure 4: Increasing incidence of fungal diseases depending on the medical advance., from Fungal Diseases: An Emerging Threat to Human, Animal, and Plant Health: Workshop Summary, 2011. Institute of Medicine; Board on Global Health; Forum on Microbial Threats; Leigh Anne Olsen, Eileen R. Choffnes, David A. Relman, and Leslie Pray, Rapporteurs. DOI: https://doi.org/10.17226/13147. Reproduced with permission from the National Academy of Sciences, courtesy of The National Academies Press, Washington, DC. (34)

Human pathogenic fungi are divided into 3 groups: primary or obligate pathogenic fungi (zootrophic fungi), fungi which first colonize humans without negative effects (the commensals) and those ones which are not related to humans or animals and belong to the natural environment (saprotrophic fungi). In Europe *Cryptococcus* is the important pathogenic fungus, *Candida* the most relevant representative of the commensals and *Aspergillus* and *Pneumocystis* the most common opportunistic saprotrophic fungi.

Opportunistic invasive fungal infection are predominant, rarely are those invasive infection of obligate fungal pathogens, which causing the disease in hosts with otherwise intact immune system (35). After invasion of the pathogenic, the eradication of the fungi and the treatment is really severe und full of complications (36). Vaccination against primary

pathogenic fungi and the opportunistic fungi does not exist at this time, but is in development (37).

Cryptococcus, *Candida*, *Aspergillus* and *Pneumocystis spp*. cause more than 90% of reported fungal infection deaths (38). Thus, invasive fungal infections (IFI) are an important cause of morbidity and mortality among patients with underlying immune dysfunction, such as those with haematological malignancies and organ transplant recipients. In this high-risk population particular invasive aspergillosis (IA) remains the dominant IFI (39).

6.2.1 *Aspergillus spp*. and invasive aspergillosis

In 1839 Johann Lucas Schönlein, a German professor of internal medicine, described the first human pathogenic fungi called *Porrigo lupinosa* (today: Tinea capitis favosa). With this publication, he was not only the first who associated a fungus with causing a human disease, but he was also the first who suspected that a microorganism can be pathogenic for humans at all (40). A student of J. L. Schönlein was Rudolf Virchow, who established the term mycoses, lectured on pulmonary fungal infections, and illustrated the dissertation of Theodor Sluyter as the first described and verified pulmonary aspergillosis in a human (41). The first description of the species *Aspergillus fumigatus* followed finally in 1863 by Georg Fresenius (42-44). The first known published drawing of *Aspergillus* is shown in figure 5.

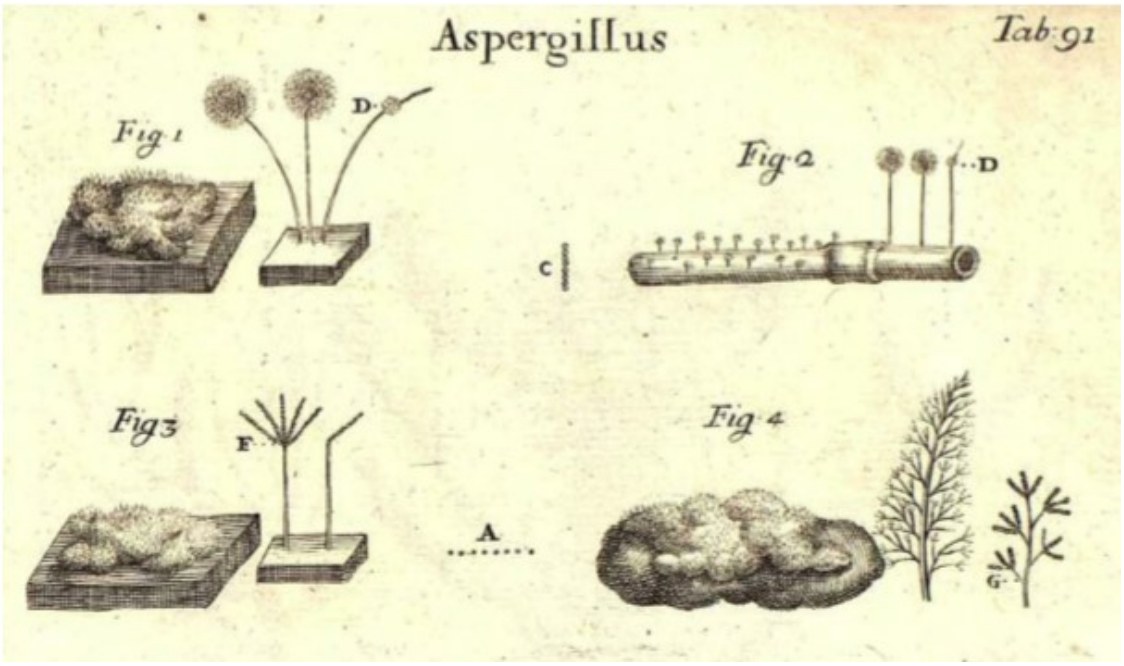


Figure 5: First known published drawing of *Aspergillus*, by Pier Antonio Micheli in "Nova Plantum Genera", 1729. Free of known copyright restrictions in the EU. PDM. No changes were made.

Aspergilli are saprophytes and occur all around the environment, e.g. in the air, the soil, organic debris and also in food. The genus *Aspergillus* comprises a few hundred species and the term *Aspergillus* derived from the Latin word *aspergere*, to sprinkle, and leans on the analogy of the sporulating head of an *Aspergillus* to the head of a water can or water sprinkler in Catholic churches, which were formerly called *aspergillum* (figure 5). *Aspergillus spp.* degrade complex polymers and recycle environmental carbon and nitrogen by secreting acids and enzymes (45). The same polysaccharide-degrading enzymes are responsible for the tissue damage in cases of *Aspergillus* infection and play a role in food processing as well as in the production of pharmaceuticals. For instance *Aspergillus oryzae* is used for the fermentation of soybeans into soy sauce, and *Aspergillus terreus* in turn for the fabrication of lovastatin, which can lower cholesterol level (46).

The spread of the ubiquitous *Aspergillus* is affected by asexual spores (conidia) through the air. Their small size makes them buoyant and enables them to reach the lung alveoli. *Aspergillus fumigatus* produces smaller conidia (diameter 2 to 3 µm) compared to other *Aspergillus* species. Simple air current is sufficient for the dispersion of its conidia; there is no complex underlying mechanism (47). Every conidial head itself produces thousands of additional conidia if enough nutrients are available (48). In- and outdoor concentration of

Aspergillus conidia ranges between 1 and 100 conidia/m³ (49). Immunocompetent humans inhale at least several hundred of these spores every day without any consequences. Of over 200 identified *Aspergillus species*, only a few are responsible for human infection in the immunocompromised patient population: *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus* and *Aspergillus nidulans*, of which *Aspergillus fumigatus* is the most prevalent and causes about 90% of the invasive aspergillosis (46).

Another point of entry of *Aspergillus* is through a breached skin barrier. In contrast to secondary cutaneous aspergillosis, which is more frequent and caused by the spreading of infection from a distal site, the initial source of primary cutaneous aspergillosis (PCA) is an infected skin lesion. With only 130 eligible reported cases between 1967 and 2015, it's a highly rare disease, although PCA is almost always lethal in immunodeficient individuals (50).

The severity of clinical consequences and developing IA after *Aspergillus* colonization of the respiratory system is ultimately determined by the type of the underlying immunosuppression of the infected host and the virulence traits of the pathogen that permit survival and germination in the pulmonary environment. The variance of fungal disease ranges between no infection, harmless colonization and self-limiting local infection, up to life-threatening angioinvasion, haematogenous dissemination and sepsis. Host defences of the fungal pathogen consist of diverse cellular functions like elimination of the evading conidia via ciliary clearance, phagocytosis, secondary inflammation and clearance of invasive hyphae. Hence a higher grade of immune dysfunction results in a higher risk for invasive infection. After inhalation, the conidia itself has to be able to evade ciliary clearance, followed by filamentous growth within the pulmonary parenchyma, penetration of the host and impairment of the cell-mediated immune defence (51).

In any healthy organism the mucociliary clearance tries to remove the conidia before reaching the pulmonary alveoli. If the first defence line is breached, facilitated due to the smaller size of *Aspergillus fumigatus* spores, phagocytosis is the next host defence line provided by the innate immune system (46). Activation of alveolar macrophages results from adhesion of the pathogen recognition receptor on the surface of the pulmonary macrophages and the molecular pattern on the surface of the pathogenic fungal conidia. Accordingly, the activated macrophages are responsible for destroying the inhaled conidia and for the production as well as the secretion of proinflammatory chemokines and cytokines such as TNF-α (secondary inflammation). These inflammatory cytokines

provoke the recruitment of the neutrophils to the site of infection and accordingly activate cellular immunity (52). Surviving conidia from the macrophage killing start germination and the transformation from conidia to the tissue-invasive hyphae. The infiltrating neutrophils are now responsible for destroying the germinating conidia and also to kill the invasive hyphae by the secretion of fungicidal proteins and reactive oxygen species (51, 53).

Various virulence traits allow *Aspergilli* to interfere with pulmonary epithelial cells, macrophages and neutrophils and to evade the immune defence lines of the innate and adaptive immune system. One immune evasion mechanism, for instance, is the secretion of extracellular proteases like Alp1, a serine protease, which can degrade collagen, fibrinogen and other extracellular matrix proteins (54). Another is the toxin production such as gliotoxin, the most produced mycotoxin of *Aspergillus fumigatus*. Gliotoxin mediates the destruction of antigen-presenting cells (e.g. monocytes and dendritic cells), and consequently inhibits the functional T-cell activation and the macrophages associated phagocytosis (55).

6.2.2 *Aspergillus fumigatus*

In contrast to other fungi *Aspergillus fumigatus* is a thermophilic species. The thermotolerance enables its survival at temperatures as high as 65°C, and conidial growth is feasible at temperatures up to 55°C. This is an essential feature for surviving on and infecting living mammals (56). In the past it was believed that the reproduction of *Aspergillus fumigatu*s is carried out just asexually, but genetic studies nowadays also describe the production of sexual stages, which offers the opportunity for genetic analysis of pathogenicity and fungicide resistance. It is still unclear as to the importance of this sexual reproductive cycle, as *Aspergilli* with known sexual stages seem to play a minor role in infection of humans (57, 58). In a large genetic study 2.026 *Aspergillus fumigatus* isolates from 13 countries were genetically analysed and it was shown that there exists a genetic diversity among geographic and ecological populations. *Aspergillus* evolution is highly influenced by localized antifungal drug selection, which can help us to better understand global rising azole resistance (59).

Although formerly thought that *Aspergillus fumigatus* is a weak pathogen simply responsible for allergic forms such as asthma, allergic bronchopulmonary aspergillosis and farmer lung, this opinion changed rapidly in recent years. The numbers of

opportunistic infections of immunocompromised patients caused by *Aspergillus fumigatus* showed a drastic increase over the last 30 years due to an increasing number of solid-organ transplant recipients, the rise of aggressive chemotherapies and other severe immunomodulating treatments (47, 49). However, pulmonary aspergillosis also occurs in immunocompetent patients with previous airway damage, such as bronchiectasis, bullous disease, sarcoidosis or tuberculosis (60). The most common manifestation of this chronic pulmonary aspergillosis (CPA) is chronic cavitary pulmonary aspergillosis (CCPA), with chronic fibrosis as a dreaded complication of its progression. Other forms are *Aspergillus* nodule and non-invasive single aspergilloma. The subacute invasive pulmonary aspergillosis deserves special attention, which affects moderately immunosuppressed individuals and should be treated like an invasive aspergillosis because of its relatively rapid progression (< 3 months). CCPA needs long term antifungal treatment to prevent chronic fibrosis, in contrast to the aspergilloma or *Aspergillus* nodule, which are difficult to access with oral antifungal therapy and surgical excision is recommended. There are estimates of more than 240.000 patients affected by CPA in Europe (61).

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity to antigens of *Aspergillus fumigatus* and occurs in patients with an underlying pulmonary disorder like asthma or cystic fibrosis. Without timely treatment ABPA can cause recurrent exacerbation, pulmonary infiltrates and even permanent lung damage and fibrotic conversion. Indeed, not all asthma patients develop ABPA despite being exposed to the same environment. It's supposed to be a polygenic disorder. The variety of unspecific symptoms results in a high rate of undiagnosed and misdiagnosed patients (62).

6.2.3 Risk factors for invasive aspergillosis

As mentioned above, the essential risk factor for invasive aspergillosis is a weak immune system, and the development as well as the severity of IA depends on the form of the underlying immune dysfunction. Host immune defence lines can be diminished on the one hand due to underlying disease and on the other hand due to the side effects of appropriate medical therapy for it, like quantitative or qualitative neutrophil defects. Neutrophils represent the dominant cells in human immune defence against infection. Morbidity and mortality increase dramatically in cases of insufficient neutrophil count (< 500 neutrophils/µl) and in patients with qualitative neutrophil defect as well (53).

Prolonged (< 500 neutrophils/µl over a duration of at least 10 days) and profound (< 100 neutrophils/µl) neutropenia induced by high dose myeloablative chemotherapy in hemato-oncological patients, like those with acute myelogenous leukaemia (AML), corresponds with the highest risk for developing IA (63). In contrast a retrospective study over a time period of ten years illustrated that only 33% of the analysed patient population with invasive aspergillosis underwent prolonged neutropenia prior to infection (64). Hence high-risk patients are not all at the same risk for invasive aspergillosis. Patients with AML and previous invasive fungal infection seem to be at a higher risk than those without prior infection. In addition AML patients who underwent stem cell transplantation may have an added risk for IFI in comparison to those who received stem cell transplantation for managing another underlying malignancy (65, 66). The constant exposure to fungus also plays an important role in hospitalized patients. Studies have shown a correlation between air concentration during the clinical stay and increased susceptibility for IA in predisposed patients (67, 68).

The 2008 defined revised diagnostic criteria of invasive fungal infections especially focused on immunosuppressed cancer patients, although numerous cases of invasive aspergillosis occur in individuals with diminished immune response due to other underlying disease (69). *Aspergillus* infection also occurs in non-immunosuppressed patients with prior chronic lung tissue damage like bronchiectasis or with chronic obstructive pulmonary disease in a less aggressive way (60). In addition, a correlation between IA onset and prolonged ICU stay, in absence of acquired immune dysfunction, was reported (70, 71). The prevalence of invasive aspergillosis in patients at the ICU, those with liver cirrhosis and those with long-term systemic corticosteroid exposure is increasing (72, 73). However, still at highest risk for IA are patients with haematological malignancies, with chronic lymphoproliferative disorders as a new high-risk group (27).

Restricted cell-mediated immune defence is caused by immunosuppressants like corticosteroids, tumour necrosis factor-α antagonists or infliximab and a low or falling level of CD4+ T lymphocytes in acquired immunodeficiency syndrome (AIDS) patients (27, 74). Immunosuppressants are of utmost importance for the prevention of life-threatening complications such as tissue rejection after solid organ transplantation (SOT) or graft-versus-host disease (GvHD) after stem cell transplantation (28, 75). Hence IA is sometimes a “collateral damage” which must be taken as a risk for managing cancer or SOT.

Hereditary chronic granulomatous disease (CGD) induces phagocytic dysfunction due to a disorder of the NADPH oxidase. The NADPH oxidase is normally responsible for producing a superoxide anion and its metabolites in phagocytic cells, and thus this patient population is highly susceptible for severe fungal infection (76). Nowadays more genetic factors are known which cause added risk factors for IA. For example, patients with mutations in some Toll-like receptors have been shown to be at increased risk. A 2009 study reported that a deficiency of mannose-binding lectin (MBL), a receptor that activates complementarily, results in the development of IA (77). However further investigations are needed to verify the role of genetic predisposition in invasive aspergillosis (78).

6.2.4 Prevention and treatment of invasive aspergillosis

In 2016 the practice guidelines of the Infectious Diseases Society of America (IDSA) for the management and diagnosis of aspergillosis were updated and published. In particular, the advance in diagnostic tools in the detection of IA without culture and new therapeutic options, including combination therapy for *Aspergillus*, were considered in comparison to the 2008 released recommendations. In this eight-year time period, the clinical data on the diagnostic performance of the newly available non-culture-based biomarkers and on the new agent options markedly increased and were incorporated into the guidelines (79).

In Europe the European Conference on Infections in Leukaemia (ECIL) published in 2017 the updated evidence-based recommendation for diagnostic strategies and targeted therapy of invasive aspergillosis in patients with underlying leukaemia and HSCT recipients. This conference is held every two years and participating societies are the European Organization for Research and Treatment of Cancer (EORTC), the European Society for Blood and Marrow Transplantation (EBMT), the European Leukaemia Net (ELN) and the International Immunocompromised Host Society (ICSH). In comparison to the last meeting only minimal modifications were made due to lack of new data and because of conducted randomized controlled trials (RCT) with only limited numbers of proven IA cases (80).

Besides antifungal treatment in suspicion of invasive fungal infection, two different therapy approaches for prevention must be distinguished: empiric treatment in febrile high-risk patients with prolonged neutropenia in spite of broad-spectrum antibacterial drugs, and the extended pre-emptive treatment in case of IA evidence (e.g. pulmonary infiltrate or

positive biomarker assay results). In the past empiric therapy strategy was introduced based on evidence due to limited diagnostic approaches of IA and high mortality rates in neutropenic fever patients with undiagnosed invasive fungal infections. The initiation of empiric treatment in allogeneic stem cell recipients and patients with underlying acute myelogenous leukaemia is strongly recommended in the context of prolonged neutropenia (duration of at least ten days) and ongoing fever despite high-dose broad-spectrum antibiotic therapy. Among the empiric antifungal agents lipid formulation of Amphotericin B (AmB) or an echinocandin (caspofungin or micafungin) are high recommended and of high-quality evidence, compared to voriconazole with moderate-quality (79). Nowadays improved diagnostic screening methods result in the replacement of empirical therapy for pre-emptive treatment. Combinations of galactomannan and PCR findings reduced the use of empirical antifungal therapy by about 52% in comparison to standard diagnostic strategy (culture and histology) (81), though no placebo-controlled trial can be found in the literature that investigated effectiveness of pre-emptive therapy (82).

The prophylactic usage of posaconazole in patients with underlying acute myelogenous leukaemia or myelodysplastic syndrome has been demonstrated to be more effective than prophylaxis with fluconazole or itraconazole since 2007 (83). In allogeneic haematopoietic stem cell transplantation (HSCT) recipients with graft-versus-host disease, posaconazole is similarly effective as fluconazole for the prevention of invasive fungal infection, but superior in prophylaxis of invasive aspergillosis (84). In comparison to the other azoles posaconazole prophylaxis resulted in a highly reduced incidence of mould infections including breakthrough infections and an improvement in overall survival (85). Voriconazole is also a prophylactic often used in clinical praxis, but it does not show any benefits in outcome (86).

The first line recommendation for prevention of aspergillosis in lung transplant recipients is prophylactic treatment with triazole like voriconazole or itraconazole for a duration of three to four months post-transplantation. An inhalative treatment with Amphotericin B may also be an option but is associated with more severe adverse events when compared to systemic triazole treatment. The reinitiation of anti-mould prophylaxis should be considered in need of immunosuppression augmentation. In cases of airway-colonization of *Aspergillus* in lung transplant patients, anti-mould prophylaxis is warranted for six months postoperative or within 3 months of intensified immunosuppression against tissue rejection. After six months and in the absence of high dose immunosuppressants and in the absence of clinical symptoms as well as radiological findings or histopathological

evidence, anti-mould prophylaxis is not recommended and should be stopped under frequent follow-up examinations.

Fewer clinical trial data are available for breakthrough invasive mould infection, however in cases of suspected or proven IA under ongoing empiric or pre-emptive treatment, a change to another mould-active drug class is indicated (79). Besides the benefit, the use of antifungal treatment in a prophylactic setting has to be well reviewed, as this can result in overtreatment with expensive antifungal drugs, the emergence of drug interactions and antifungal resistance (87). A study involving eight hospitals in the Netherlands detected geographical migration of voriconazole resistance in *Aspergillus* isolates (88). Despite the differentiation between primary (intrinsic) and secondary (prior azole exposure), the most frequent causing mechanism is the same: mutation or overexpression of target enzyme CYP51A (89).

If findings indicate a suspected invasive aspergillosis, the use of voriconazole is recommend as first line treatment. A loading dose of voriconazole of 6 mg/kg two times on day 1 is suggested, followed by 4 mg/kg twice daily. Treatment of IA should be applied at least 6 to 12 weeks depending on the duration and dose of immunosuppression, the site of disease and the clinical progression (79, 80). In a recent randomized-controlled study isavuconazole as a first line therapy seemed to be as effective as commonly used voriconazole and had less adverse effects (90). Alternative first line treatments include liposomal Amphotericin B (91). Amphotericin B deoxycholate plays no role as a standard therapy anymore due to its lower effectiveness and to its poor safety profile (79, 80, 91). Lipid formulations of AmB are just used in salvage treatment settings and in the absence of voriconazole isavuconazole or triazole are contraindicated (80). Several trials and post hoc analyses comparing voriconazole versus AmB did not change the liposomal Amphotericin B therapy recommendation over the years (92-94). A randomized, double-blind, placebo-controlled multicentre study in 2015 analysed a combination therapy of voriconazole with anidulafungin (echinocandin) versus voriconazole with a placebo in the first line treatment. Initial treatment with an echinocandin itself in the case of IA is contraindicated. This study didn’t reach the primary endpoint of a decreased all-cause mortality at week 6, though ultimately the subgroup analysis showed a higher survival for the patients receiving first-line combination antifungal therapy. Therefore, the finding led to a weak recommendation for combination therapy, but it is not routinely recommended in the absence of more well-designed studies (79, 80, 95). An overview of the ECIL 6 recommendation in the first line is shown in table 1.

Antifungal	Grade	Remarks
Voriconazole	A I	Day 1 2x6mg/kg, then 2x4mg/kg
Isavuconazole	A I	Day1+2 3x200mg, then 200mg daily dose
Liposomal Amphotericin B	B I	3-5mg/kg daily dose
AmB lipid complex	B II	5mg/kg daily dose
AmB colloidal dispersion	C I	
Caspofungin	C II	
Itraconazole	C III	
Combination voriconazole + Anidulafungin	C I	
Other combinations	C III	

Table 1: ECIL-6 recommendations in the first line (80).

Infrequently surgical intervention can be used as an additional therapeutic option in the case of extrapulmonary accessible tissue affection of IA but it is limited due to the risk of bleeding and other complications in this often critically ill patient population. The primary intention is the recovery of histopathological material for diagnosis and antifungal susceptibility testing, however surgical debridement can also decrease *Aspergillus* burden by eliminating already necrotic tissue, which can't reached by anti-mould active therapy (96).

6.3 Diagnosis of invasive aspergillosis

In the early beginning only the microscopic identification on the basis of phenotypic characteristics of the pathogen was possible. Given the variance of *Aspergillus* species and their variable pathogenicity and antifungal susceptibilities, better diagnostic approaches were urgently needed. In the last 20 years the diagnostic options of invasive fungal infection increased enormously and provided faster and more precise results.

The European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group (EORTC) and the Mycoses Study Group of the National Institute of Allergy and Infectious Disease (MSG) defined revised criteria for the diagnosis of invasive fungal infections in 2008 (69). EORTC/MSG criteria strongly depend on mycological evidence and on histopathological proof of fungal tissue invasion for the definite diagnosis of invasive aspergillosis (69). However, the needed invasive specimens' acquisition is often not feasible in critically ill patients due to procedure-related life-threatening complications like uncontrolled bleeding or additional infection. In cases of suspicion of invasive pulmonary infection, tissue sampling would imply bronchoscopy with transbronchial and/or endobronchial ultrasound guided biopsy, CT-guided transthoracic needle biopsy or even video-assisted thoracoscopic biopsy in the most severe immunosuppressed patients. Therefore, nowadays non-invasive diagnostic methods are applied in clinical routine analysing samples which can be more easily obtained, such as blood, sputum and bronchoalveolar lavage fluid, and also urine (97-101).

Correct interpretations of the test results are essential before the determination of the clinical consequence and should include patients' parameters and characteristics of the used diagnostic method. Established diagnostic guidelines improve and facilitate the diagnostic process and the resulting clinical decision such as the initiation of antifungal treatment (69, 79, 80). According to the EORTC/MSG criteria the majority of non-fatal IA cases are classified as probable disease, which requires fulfilment of predefined host factors, clinical criteria and mycological criteria that include fungal culture (69, 102). Combination of classical clinical symptoms and risk factors without direct or indirect detection of *Aspergillus* is scaled as possible IA. This graduation of probability of invasive aspergillosis in possible, probable and proven is always indicated and the basis of every clinical consequence in the treatment of invasive aspergillosis (EORTC/MSG criteria are shown in table 2).

Proven IA (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 melanised 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 IA (host, clinical and microbiological criteria must be met)

- Host criteria:
  - Recent history of neutropenia (<0.5x109 neutrophils/L) for >10 days
  - Receipt of an allogeneic stem cell transplant
  - Prolonged use of corticosteroids at a mean minimum dose of 0.3 mg/kg/day of prednisone equivalent for >3 weeks
  - Treatment with other T-cell immunosuppressants such as cyclosporine, TNFα 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 lesions(s) with or without a halo sign
  - Air-crescent sign
  - Cavity
- Tracheobronchitis
  - Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopy
- Sinonasal 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

  - One of the following two signs:
    - Focal lesions on imaging
    - Meningeal enhancement on MRI or CT

→ <u>Mycological criteria (one of the following must be met)</u>

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

Possible IA

Fulfilled host factors and clinical criteria for IFI, but absence of mycological criteria

Table 2: 2008 revised EORTC/MSG criteria.

6.3.1 The role of imaging

Pathogen conidia frequently affect the lungs since the primary route of infection is inhalation. High-risk patients with prolonged or profound febrile neutropenia should get chest imaging to identify the site of infection. A chest x-ray is not sensitive enough for diagnosis of invasive aspergillosis (figure 6), and in cases of additional symptoms like dyspnea or chest pain, chest computer tomography (CT) is indicated. Early chest CT is associated with a better outcome in haemato-oncological patients with febrile neutropenia after chemotherapy and stem cell transplantation (103, 104). Magnetic resonance imaging (MRI) of the chest does not give any additional information in cases of IA suspicion and usually has a longer waiting period in clinical settings. In today's relevant guidelines a

high-resolution CT scan (layer thickness of 0.25–1 mm) of the chest is strong suggested, regardless of chest x-ray results. Application of contrast during a chest CT scan is not obligatory and depends on the decision of the attending physician. Clinical symptoms of the patients like haemoptysis and consequently possible lung vessel complication should be considered (79). Nonetheless computer tomography pulmonary angiography (CTPA) is more sensitive and improves the diagnostic value of CT imaging in patients under suspicion for invasive mould infection (105). Chest CT scans play not only an important role for the evaluation of the type, number and dimensions of the lesions, it is moreover an essential tool in direct diagnostic methods such as CT-guided percutaneous needle aspiration for peripheral lesions (106). Typical chest CT findings in cases of invasive pulmonary aspergillosis (IPA) consist of well circumscribed nodules or consolidation, air crescent sign, cavity formation (figure 7) in a mass and, particularly in patients with neutropenia, a halo sign (69). A halo sign is specified as a nodule with a diameter of at least 1 cm surrounded by ground-glass grey attenuation (figure 8), which represents central tissue necrosis and surrounding haemorrhage and can be seen clearly only during the first 10 days of angioinvasion (107, 108). An air crescent sign is defined as a nodule or consolidative lesion surrounded by a crescent-shaped area of radiolucency and is an often-reported radiologic pattern in cases of IPA. An air crescent sign or a cavity formation is seen mostly in a later point of infection and could be a sign of recovery of immune defence and neutrophil count (107, 109). After initiation of antifungal therapy, a follow-up CT scan of the chest should be initiated at the earliest two weeks after treatment start unless probable lung vessel involvement and risk for fulminant haemoptysis (79). Caillot et al. published a lesion’s growth is up to fourfold during the first week, and a steady state in the second week after the onset of anti-mould therapy. Hence a chest CT follow-up earlier is not warranted with the exception of the clinical aggravation of the treated patient (110).

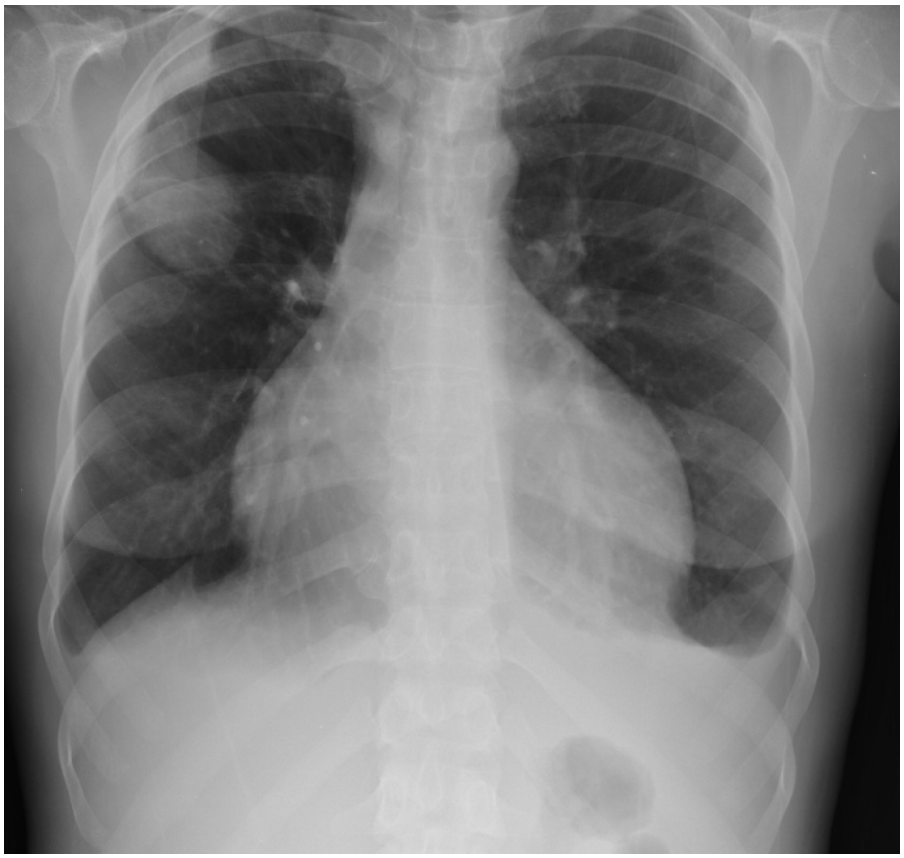


Figure 6: X-ray of an aspergilloma in the right upper lung, by Yale Rosen, distributed by Creative Commons Attribution-Share Alike 2.0 Generic license. No changes were made.

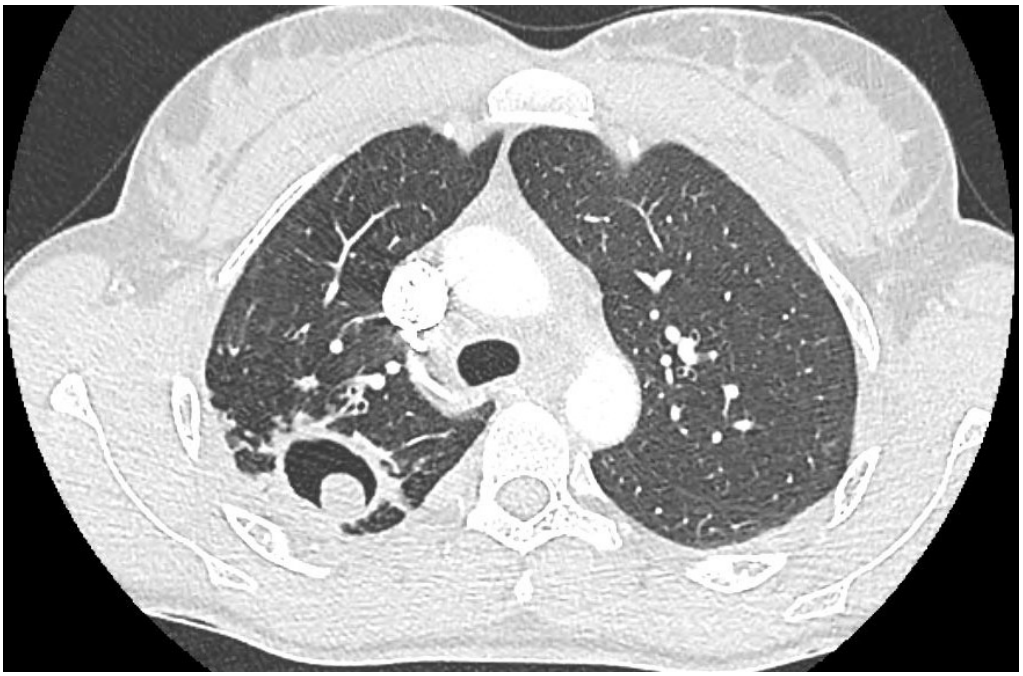


Figure 7: Chest CT ray of an aspergilloma in the right upper lung, by Stockholm, distributed by Creative Commons Attribution-Share Alike 4.0 International license.

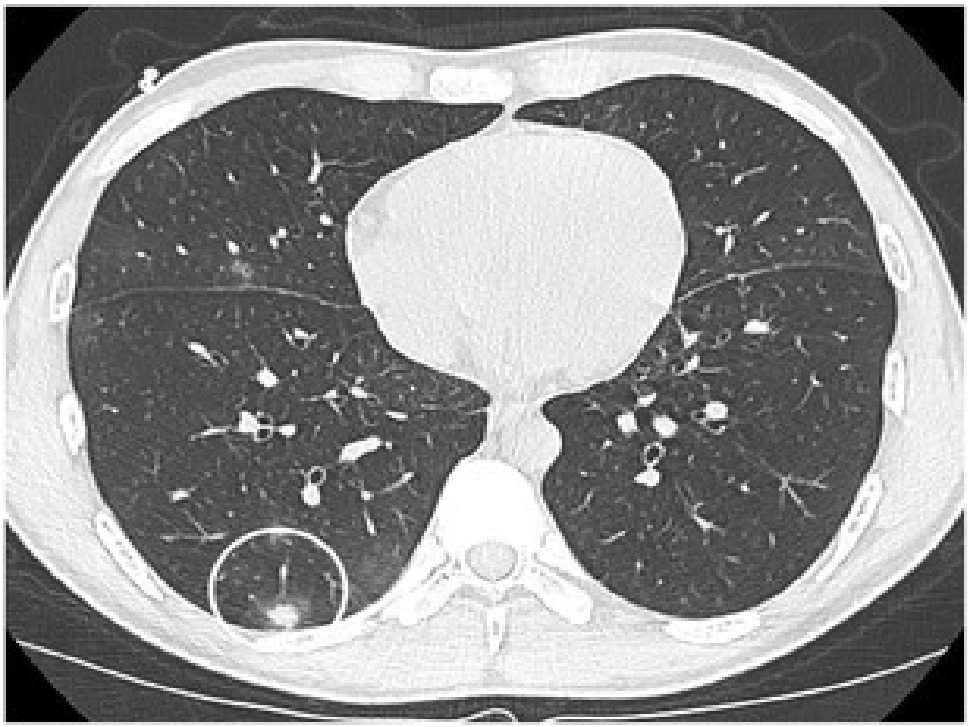


Figure 8: Nodule with surrounding ground glass halo in the in the right lower lung, by Snoeckx et al., distributed by Creative Commons Attribution 4.0 International license (111).

6.3.2 Histopathology and *Aspergillus* culture

According to the revised EORTC criteria proven aspergillosis requires histopathological evidence of *Aspergillus* by direct isolation from the site of infection (69). Affected lung tissue specimens are mostly obtained via sterile procedure like CT-guided transthoracic needle biopy or video-assisted thoracoscopic biopsy when the overall state of the patient allows. Independent of the executed procedure enough quantity is needed for concurrent histopathologic/cytologic and culture examination. Timely delivery of the samples to the analysing laboratory or specimen refrigeration under special circumstances and an incubation period of culture not less than five days plays an important role in diagnostic accuracy (79).

Special fungal stains, like fast and highly sensitive Calcofluor or Blankophor, should be used for detection of the characteristic 45 degree angle septate hyaline hyphae under the microscope (112), shown in figure 9. With these rapid optical brighteners tissue specimens can be directly dyed. Gomori methenamine silver and periodic acid-Schiff are used after fixation of the sample. Nonetheless the diagnostic accuracy of histopathologic

and cytopathologic examination of IA is low, and also limited by long turn-around time and lack of sensitivity (113, 114). Additional culture, other diagnostic techniques and molecular methods are needed for the differentiation of *Aspergillus* from other filamentous fungi (e.g. *Fusarium spp*. and *Scedosporium spp.*) due to their similarity to other causative fungal pathogens and can't be made by histopathologic findings alone (115).

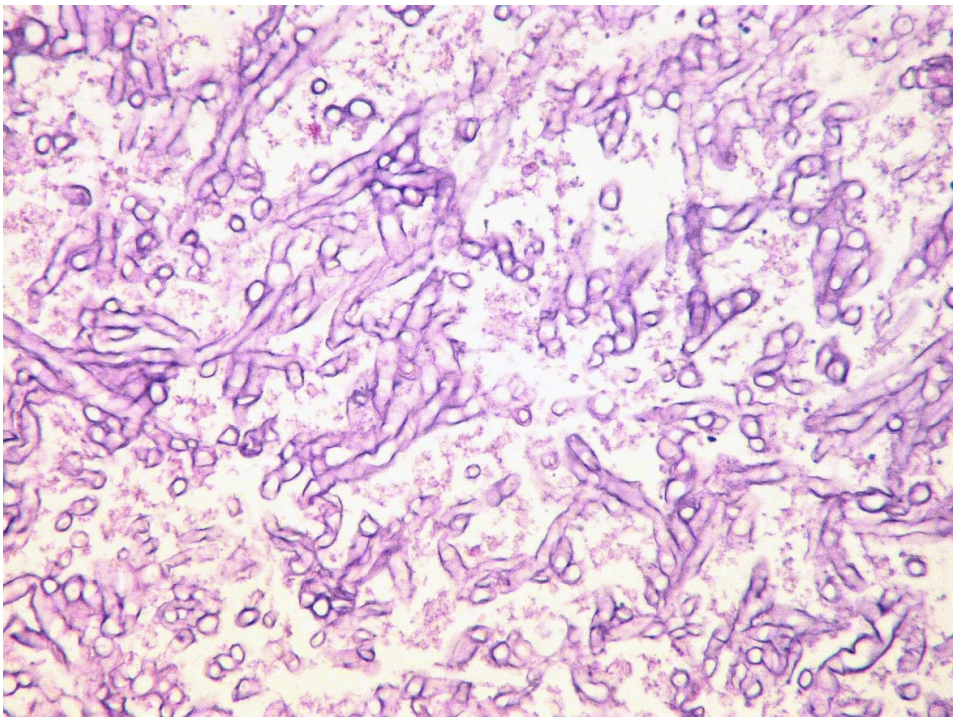


Figure 9: Typical *Aspergillus* hyphae exhibit dichotomous 45-degree angle branching and septae, by Yale Rosen, distributed by Creative Commons Attribution-Share Alike 2.0 Generic license. No changes were made.

Temperatures between 30 to 37°C are ideal for the growth of *Aspergillus* on most media. Typically, *Aspergillus fumigatus* shows high growth rates and the sporulating head develops within 24–48 hours on specific fungal media as wells as on standard culture media such as blood agar or chocolate agar, which is primarily used for bacterial verification. Specific media for fungal culture compromises among others malt extract agar, glucose agar (figure 10) and brain heart infusion agar, and can lead to faster growth in rats (116, 117). But species identification is often challenging. Balajee et al. illustrated a less sporulating white strain of *Aspergillus fumigatus*, which develops the typical blue-green sporulating not before 10–12 days of incubation (118). A negative culture result

after an incubation time of at least five days does not exclude the existence of IA, and besides culture is not appropriate for susceptibility testing (119). In contrast due to the constant exposure of *Aspergillus* in nature a positive culture result is not automatically associated with the presence of IA and may reflect only colonization of the respiratory system or could be caused by laboratory contamination as well (116). A wide range, between 14% and 72% for the positive predictive value (PPV) of lower respiratory tract cultures in immunodeficient patients, was reported, with the highest PPV in stem cell recipients (120). Hence fungal culture is often time-consuming and moreover poorly sensitive (121). Culture results should never be interpreted alone for the diagnosis of invasive aspergillosis. For a better assessment the presence of clinical symptoms and risk factors, as well as radiologic findings for invasive aspergillosis and additional positive culture results from other sites of the same patient if available, must be considered. Though microscopy and culture remain essential tools, nowadays the DNA sequencing is getting more and more relevant for fungal pathogen identification in clinical routine (116).

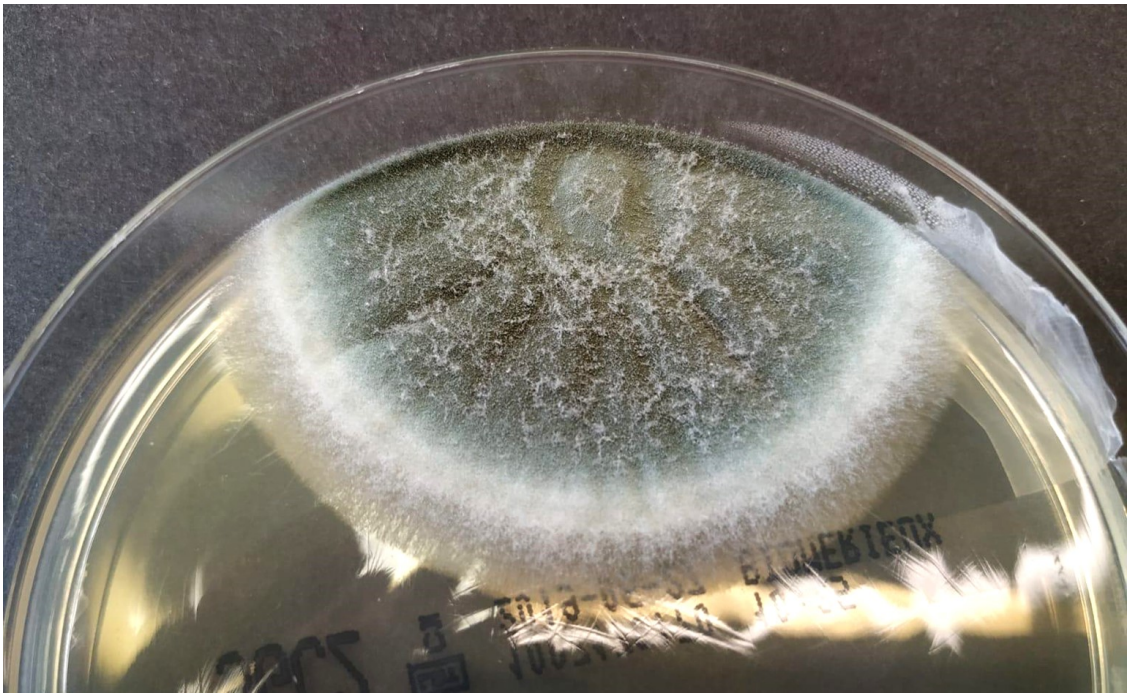


Figure 10: *Aspergillus fumigatus* culture. Growth after two days on a sabouraud dextrose gentamicin chloramphenicol agar.

6.3.3 The role of biomarkers for diagnosis

Rapid detection and early start of mould-active therapy are key factors in the successful treatment of invasive mould infections (IMI). Clinical signs and symptoms of IA like fever as well as radiological findings, are often unspecific in the early phase of disease (122-124). IA is a major threat to humans, with high mortality rates due to the absent and limited capabilities for timely diagnosis. Various studies have shown that early initiation of antifungal therapy crucially improve survival rates (107, 122).

In the last years the indirect detection of *Aspergillus spp.* through biomarker tests, with rapidly available test results, has become an important cornerstone of mould infection diagnostics (125). Proteins, called antigens, are on the cell surface of all pathogens; the human immune system can recognize these antigens and respond in the form of antibodies to destroy the foreign microorganism. Antigen tests rely on the fact that there is a specific antibody for each antigen, which enables early diagnosis, pre-emptive treatment and thereby the improvement of overall survival in patients with invasive aspergillosis (126, 127). Furthermore, antigen testing also prevents overtreatment which has become frequent, and hence reduces health care costs and the development of drug resistance (128). Finally, antigen testing also may be useful for early response assessment, therapy monitoring and treatment stratification, including the decision of when to stop antifungal therapy in patients with underlying invasive mould infection.

In combination with *Aspergillus* culture, antigen test results improve specificity and sensitivity of fungal culture alone and can be performed on body fluid or out of affected tissue samples. As mentioned above invasive diagnostic procedures for tissue sample recovery is often life-threatening in high-risk patients with low platelet counts and severely affected respiration. Thus, in clinical routine the easier and less risky way is to take body fluid specimens like blood, bronchoalveolar lavage fluid (BALF), pleural fluid or cerebrospinal fluid samples and urine depending also on the site of infection. Meanwhile established biomarkers in daily clinical routine are galactomannan (GM) and 1,3-β-D-glucan (BDG), which use different modes of detection. Numerous and varying studies about the diagnostic performance of this tests were published; nonetheless further investigations of newer biomarker tests for reliable and fast diagnosis of invasive mould infections are needed (99, 129).

6.3.3.1 Galactomannan (GM)

Galactomannan (GM) is a polysaccharide cell wall component of *Aspergillus spp.* that is released into the circulation only by growing hyphae and germinating spores. In case of IA-suspicion GM is measurable in peripheral blood (serum and plasma), bronchoalveolar lavage fluid (BALF), urine, cerebrospinal fluid and/or pleural fluid (99-101, 130). However the test also has limitations like false positive results, factors such as co-medication (e.g. β-lactam antibiotics) and former medication of the patient, underlying diseases and host factors (e.g. renal failure), and also cross reaction with food and bacteria must be considered for the correct interpretation of GM test results (86, 131-133). In addition the sensitivity of serum GM decreases significantly in cases of antifungal prophylaxis or empirical/pre-emptive therapy, whereas sensitivities of nearly 80% were reported in case of prophylaxis or empirical therapy (134-136). GM may therefore be useful for diagnosing breakthrough infections in cases of antifungal prophylaxis or empirical therapy. Another limitation is that GM results are not always rapidly available, as the time to result is 48 hours or more in many clinical settings, which can cause a potentially fatal delay of treatment. Other fungi, which also secrete GM or other cross-reacting antigens like *Fusarium spp.* or *Penicillium spp.*, can cause false positive GM results in cases of infection (137, 138).

Serum GM detection by the Platelia enzyme-linked immunosorbent assay (ELISA) has proven to be a promising tool for the diagnosis of IA over the years and is FDA (U.S. Food and Drug Administration) approved for BALF and serum specimens. Previously used Latex agglutination was replaced because of low sensitivity (139). GM results were displayed as the optical density index (ODI), a ratio of the optical density of the analysed sample and the optical density of the control of each test kit. Higher cut-offs were used in the beginning of GM testing, which led to high rates of false positive test results. Studies investigating the diagnostic performance of GM reported improved performance when lower cut-offs were used (140, 141). Twenty-seven studies between 1966 and 2005 were included in a meta-analysis, which depicted a sensitivity of 71% and a specificity of 89% for the double-sandwich enzyme-linked immunoassay for proven invasive aspergillosis. Remarkable heterogeneity was reported given the diverse patient population, the used reference standard type and the used cut-off values (142). In a prospective analysis serial screening for circulating galactomannan was performed and positive GM results were seen before the beginning of clinical symptoms or the occurrence of radiologic findings in patients with proven invasive aspergillosis confirmed after autopsy. High sensitivity of 89.7% and a specificity of 98.1% were reported (143). So, serial screening for GM can be

a highly sensitive tool for early diagnosis and rapid initiation of antifungal treatment in patients at risk for IA. This prophylactic therapy strategy lowers the incidence of invasive aspergillosis in an immunosuppressed patient population, but also lowers the sensitivity of GM screening result (134-136). Besides the screening GM detection is also used for therapy monitoring in patients with proven IA. Persistent positive GM values under ongoing mould-active treatment in patients with proven and probable invasive aspergillosis seem to correlate with higher mortality rates (144). Accordingly falling GM values in invasive aspergillosis patients indicated better overall survival (145).

Before GM introduction among the pathogens identified Candida spp. were most frequent (146); after the introduction of GM testing invasive mould infections diagnoses increased. Responsible for the shift from possible IA, meaning causative pathogen remains unknown, to probable IA was the GM testing by detection of mycological evidence in most of the probable cases in 2010 (127). The use of serum GM offers a prognostic marker in patients with IMI and is a valuable tool for treatment stratification and can reduce unnecessary antifungal therapy (79). GM serum testing (cut-off 0.50 ODI) is routinely performed twice weekly in all patients with haemato-oncological disease at risk for IA at our institution.

Diagnostic performance of GM in bronchoalveolar lavage fluid (BALF) specimens seems to be more promising than in serum (147) and has been established in clinical routine in haemato-oncological centres throughout the world (106, 148). The ideal cut-off defining a positive test result, however, is still a matter of dispute, as lower cut-offs lack specificity, whereas higher cut-offs lack sensitivity. Indeed, BALF can sometimes not be obtained, as bronchoscopy is hindered by the severe condition in these critically ill patients at risk for IA. GM testing at our institution is routinely performed in all BALF samples from patients with haemato-oncological disease with a cut-off 1.00 ODI.

Measurement of GM index in urine specimens may provide several advantages: (a) sample collection is non-invasive and easy, hence providing possibilities of home testing in the future, and (b) in contrast to serum and BALF large volumes may be obtained and examinations could be more frequent, which may increase sensitivity of the GM test. Reischies et al. evaluated serial urine samples from 71 patients with underlying haematological malignancies and investigated the urine GM/creatinine ratio, i.e., (urineGMlevel100)/urine creatinine level, which takes urine dilution into account. They illustrated that GM urine levels correlate with those obtained in serum in patients and reliably detected IMI. Urine GM may be a promising additional diagnostic tool (101).

6.3.3.2 1,3-β-D-glucan test

Beta-D-glucan (BDG) is another polysaccharide cell wall component of most fungal pathogens (e.g. *Aspergillus spp.*, *Candida spp.*, *Pneumocystis jirovecii*, *Fusarium spp.*), except *Zygomycetes spp.* and *Cryptococcus spp*. (134, 135, 147-150). Since 2008, the serum 1,3-β-D-glucan test has also been a diagnostic criterion for invasive fungal infection in the revised European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) guidelines (151) and has been established in clinical routine of many haematologic centres in Europe and the United States. The commonly used Fungitell® assay (Associates of Cape Cod, Inc., East Falmouth, MA, USA) is FDA approved and measures BDG based on the influence of the coagulation cascade in the Limulus amebocyte lysate, a horseshoe crab substrate (152). Ostrosky-Zeichner et al. found high sensitivity and specificity for the serum 1,3-β-D-glucan in a multicentre controlled study conducted in six hospitals in the United States. The sensitivity and specificity estimated in that protocol were 70% and 87% respectively (153). In general, β-D-glucan detection is less sensitive and specific for invasive aspergillosis compared to GM testing. The BDG test results positive also in invasive Candida infections, Pneumocystis and various other fungal infections (154, 155). BDG values were defined as positive over 80 pg/mL and as negative under 60 pg/mL for patients with haematological malignancies at risk for invasive candidiasis and early detection of breakthrough infection. Consequently the ideal cut off level for invasive aspergillosis may differ (156). However, the practice guidelines recommend the additional 1,3-β-D-glucan testing in patients at risk for invasive aspergillosis (79). At our institution β-D-glucan serum measuring is performed together with GM testing twice weekly in patients at risk for IFI (cut-off 80 pg/mL).

Only limited data currently exist on the performance of 1,3-β-D-glucan testing in BALF (29). Prattes et al. showed that the β-D-glucan assay, when tested in BALF, was limited by a low specificity varying from 42% to 61% (99). The specificity has been described as the major limitation of the BDG assay also in other smaller studies (157, 158) due to the fact of potential false positivity Candida colonization. Indeed, the high NPV (up to 97% using the 80 pg/ml cut-off) may be useful to rule out clinical suspicion of invasive pulmonary aspergillosis (99).

Less is known about the BDG measuring by Fungitell® in other body fluids like urine or cerebrospinal fluid. In a recent study same-day midstream urine and serum samples were collected in adult patients with underlying hemato-oncological malignancies and a significant positive correlation between BDG in serum and urine samples was observed.

Urine BDG detection may be promising only in case of high-positive urine results, which correlated well with high-positive same-day serum BDG results (159).

6.3.3.3 Lateral Flow Device Test (LFD)

One of the major limitations of the GM test is that time to results varies significantly among specialized centres, from less than a day up to several days. This mainly depends on the number of specimens tested in succession, as the test is performed with plates covering 96 tests, and the distance/duration of transport between the clinic and the laboratory where the test can be performed. The Lateral Flow Device Test overcomes these limitations, a point-of-care test developed for the diagnosis of invasive mould infection by C. Thornton of the University of Exeter, United Kingdom, based on detection of a monoclonal antibody (MAb JF5) secreted during the active growth of *Aspergillus fumigatus* (160). This point-of-care test can be easily performed in every laboratory using BALF and/or serum specimens after minimal required training without any bigger effort or much previous knowledge. Furthermore, the LFD provides accurate and quickly available results (approximately 20 minutes or less), which, combined with low costs, are the major advantages of the LFD. Studies have shown the potential of the test in human BALF and serum samples (97, 98, 160). Therefore, this new single-sample test may be a very promising diagnostic approach for detecting invasive pulmonary aspergillosis in BALF and serum specimens from haematological malignancy and SOT patients. In an animal model of IA it was shown that performance of the BALF LFD was not strongly influenced by systemic antifungals (97). Little is known, however, about the influence of systemic AF prophylaxis and therapy on the performance of the LFD in human BALF samples. Prospective studies are needed to further evaluate the point-of-care LFD test.

6.3.4 Polymerase chain reaction for invasive aspergillosis

Besides these antigen tests the polymerase chain reaction (PCR) techniques have been developed in the recent years for better diagnosis of invasive fungal infections. PCR has been shown to be a very promising method for the detection of IFI (161-165), especially

when combined with the GM test and performed in the earlier stage of fungal infection. It remains unclear whether these tests need to be performed with BALF (i.e., primary site of infection) or whether the testing of single blood samples is sufficient to achieve acceptable sensitivity. However, a lack of standardization or clearance by the FDA for clinical use, are main limitations of PCR diagnostics. Harmonization efforts are ongoing to enable inclusion of PCR into the next revision of the EORTC classification (79, 166).

The performance of *Aspergillus* PCR is promising in BALF where sensitivities and specificities above 90% have been reported (167-169). Another study from Mannheim reported sensitivity and specificity of 86% (95% confidence interval [CI], 65% to 95%) and 100% (95% CI, 86% to 100%) when using pleural effusion and biopsy samples (165), while the majority of studies has reported PCR as being useful for detection of *Aspergillus* in blood specimens (161), and also in combination with the LFD test (162). Sensitivity and specificity of *Aspergillus* PCR on BALF were reported as 77% and 94%, respectively, in a review that included nine studies (170). With PCR assay the differentiation between patients suffering from the colonization of the respiratory tract and those suffering from invasive disease is not possible, but the high negative predictive value (≥ 95%) can rule out invasive pulmonary infection. Diagnostic performance of BALF PCR was comparable to BALF GM, and both together showed an improved sensitivity without decreasing specificity (170). Moreover, the combination of PCR and GM reduced the use of antifungal therapy and enabled earlier diagnosis (81, 171) .

Panfungal PCR is essential for detecting rare fungal species, which are misidentified as microscopics like *Mucorales, Fusarium spp.*, *Scedosporium apiospermum*, and *Rhizopus spp.* (172). Babouee et al. reported in 2013 that panfungal PCR from biopsy specimens revealed 100% sensitivity on a patient level for proven invasive fungal infection (173).

The application of PCR using blood specimens is still being discussed. On the one hand PCR shows promising results used in combination with other methods, e.g. antigen tests, for the diagnosis or control of antifungal treatment, but on the other hand the incongruity of the available assays, the lack of conclusive validation and the dubiety of the test result interpretation are the reasons why PCR is still not recommended for routine use in clinical practice (79). In 2014 a meta-analysis of twenty-five studies with 2.595 patients reported sensitivity of 84% and specificity of 76% when using serum or whole-blood PCR assays for the diagnosis of invasive aspergillosis in haematological patients (174).

7 Aim of this Study

The aim of this study was to evaluate biomarkers for diagnosis of invasive aspergillosis: established biomarkers like Galactomannan, which is already used as a standard diagnostic test, and novel diagnostic approaches like *Aspergillus* DNA detection by A*spergillus* specific PCR and Panfungal PCR, and combinations of these biomarkers by using BALF samples and blood samples obtained on the same day (within 24 hours) in patients with haematological malignancies at risk for invasive mould infection (175).

In cooperation with the University Hospital of Mannheim, Germany, we examined a multifungal DNA microarray developed at the University of Mannheim that detects a variety of clinically relevant fungal pathogens (later also referred to as panfungal PCR) (176), as well as *Aspergillus*-specific PCR, Galactomannan in all BALF and biopsy samples in addition to corresponding whole blood samples. To our knowledge this represents the first study that evaluates this extensive panel of diagnostics in BALF and same-day blood samples (175).

In addition we evaluated BALF LFD performance in a subset of the prospectively enrolled cases with haematological malignancies, performed an analysis of BAF LFD performance in a mixed cohort of immunocompromised patients and evaluated the influence of mould-active antifungal treatment on the performance of the LFD in patients with invasive aspergillosis (177, 178).

8 Material and Methods

8.1 Study Design

For this study all patients above 18 years of age with underlying haemato-oncological malignancies admitted to the University Hospital Graz, Austria, were screened for inclusion. In cases of a risk for invasive aspergillosis and performance of a bronchoscopy with a diagnostic BALF recovery, patients were finally included into analysis after informed consent was obtained. The study period was 20 months for sample collection at the Medical University of Graz, from April 2014 to November 2015. The study flow is displayed in figure 11 (175).

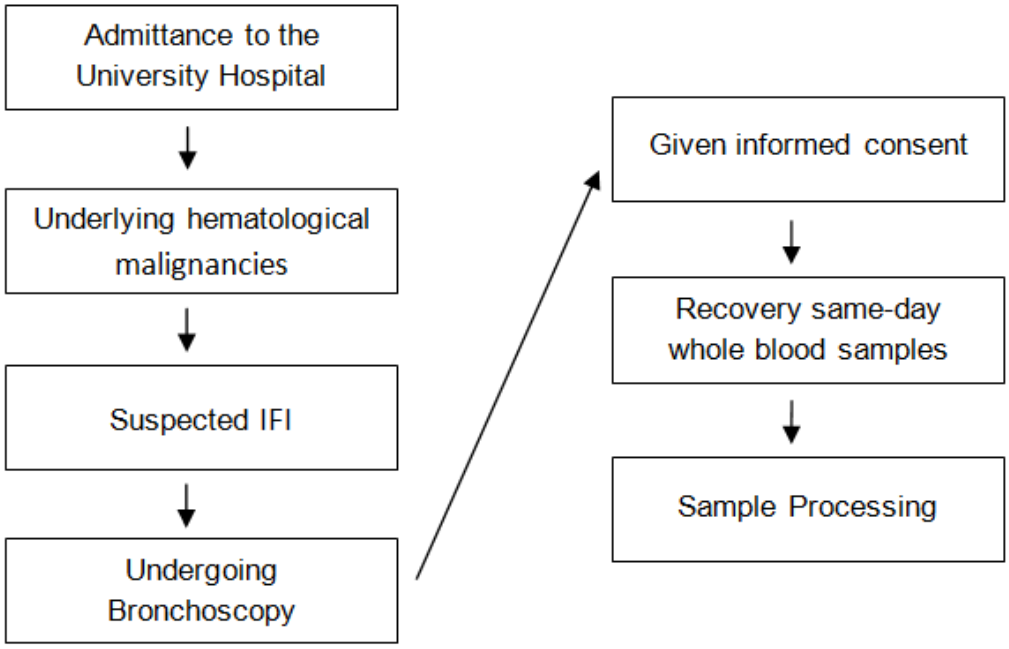


Figure 11: Study flow at the Medical University of Graz.

Invasive *Aspergillosis* is a rare disease and due to the broad usage of mould-active antifungal prophylaxis in those at highest risk for developing to disease, it is extremely challenging to obtain enough cases for the calculation of the sensitivity of tests in prospective single-centre studies. We therefore decided to expand our cohort by including cases from Mannheim, Germany, which were enrolled retrospectively.

Conventional mycological culture was routinely and prospectively performed in Graz (Microbiology Laboratory, Department of Internal Medicine) and Mannheim (Institute of Medical Microbiology and Hygiene, Mannheim University Hospital). BALF and serum GM concentrations were also prospectively measured in clinical routine by the Platelia EIA (Bio-Rad Laboratories) in Graz (Institute of Hygiene, Microbiology and Environmental Medicine until October 2014, afterwards at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Austria) and Mannheim (Institute of Medical Microbiology and Hygiene, Mannheim University Hospital). At the Medical hospital Graz all BALF aliquots together with the whole blood sample were initially stored at 4°C and finally shipped overnight to the scientific laboratory of the University Hospital of Mannheim, Germany for BALF *Aspergillus*-specific and panfungal PCR testing (175). Nested *Aspergillus* PCR assay was performed prospectively in all study samples of Graz (and within 48–96 hours of sample collection) according to the protocol of Skladny et al. (175, 179). Extraction of DNA was processed via phenol-chloroform extraction according to the protocol of Sambrook et al (175, 180).

8.2 Patient enrollment

Clinical rounds and chart reviews at the wards in Graz were performed daily to enable early study inclusion. Data including antifungal therapy, underlying diseases, chemotherapy, stem cell transplantation, host factors for IFI, concomitant medications, microbiologic test results, radiological findings, blood count, results of routinely performed antigen tests, all-cause mortality after 30 days and after 90 days, and causes of death if applicable, were collected of all patients. Patients names were anonymised with a continuing code (175).

8.2.1 Inclusion criteria

Key inclusion criteria were (a) above 18 years of age, (b) admittance to the University Hospital of Graz, Austria, (c) underlying active haemato-oncological malignancy,(d) at risk for IA according to attending clinicians. Risk factors included febrile neutropenia, induction chemotherapy for acute myeloid leukaemia, allogeneic stem cell transplantation/graft-versus-host disease, prolonged high-dose corticosteroid therapy and having clinical/radiological/mycological findings suspicious for IFI. All the patients have (e) to give informed consent to be included. An overview of the key inclusion and exclusion criteria is shown in table 3. All patients who fulfilled the inclusion criteria between April 2014 and November 2015 at the Medical University of Graz and signed informed consent were included in the prospective analysis (175).

Inclusion criteria	Exclusion criteria
Above 18 years of age	Under 18 years of age
Admittance to the University Hospital of Graz	Not admitted
Active haemato-oncological malignancy	No underlying active haemato-oncologic malignancy
Risk for IA according to attending clinicians	Not at risk for IFI
Give informed consent	Informed consent not given

Table 3: Overview of the key inclusion and exclusion criteria (175).

8.2.2 Patient enrollment in Mannheim

Patients with available same-day BALF and blood samples from July 2013 to May 2014 and suspected IA were enrolled retrospectively at the Mannheim University Hospital, Germany, for better test performance interpretation. According to the inclusion criteria 15 patients with mixed underlying diseases (12 patients with haematological malignancy and three patients with other underlying diseases) were included in the final study (175).

8.2.3 Patient classification

Patients were classified as having proven, probable, or possible IA, or no IA in accordance with the revised criteria by the European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group (EORTC)/Mycoses Study Group (MSG) of the National Institute of Allergy and Infectious Disease with the inclusion of BALF GM > 0.5 ODI as mycological criteria. All patients with possible IA and those who did not have GM and PCR results from BALF, and same-day blood were excluded from the final analysis (175).

8.3 Diagnostic management in cases of suspected IA

8.3.1 Clinical routine procedures

Chest CT is routinely performed promptly in all patients with suspected invasive pulmonary aspergillosis (the majority happen on the same day) at our institution. Bronchoscopy with BALF recovery and/or biopsy is performed in most patients with CT findings suggestive of IMI depending on the clinical condition of the patient (e.g. severe respiratory detraction, sufficient platelet count). Serum screening for Galactomannan is routinely used and is carried out twice a week in patients at risk for invasive fungal infections at the haematology ward in clinical routine. In patients at the bone marrow transplant (BMT) unit serum screening is even performed three times a week. If bronchoscopy is possible GM testing is routinely performed in all BALF samples, in addition to microscopy and conventional BALF culturing.

8.3.2 Study related procedures

In cases of bronchoscopy BALF aliquots were reserved for study purposes in addition to whole blood samples that were collected on the same day (maximum 24 hours according to bronchoscopy). BALF (or biopsy/pleural effusion) aliquots, together with the whole blood samples, were shipped overnight to a scientific laboratory in Mannheim, Germany for BALF *Aspergillus*-specific and panfungal PCR testing. Routine and study-related procedures at the Medical University of Graz concerning biomarkers/diagnostics in serum and BALF specimens are displayed in Figure 12 (175).

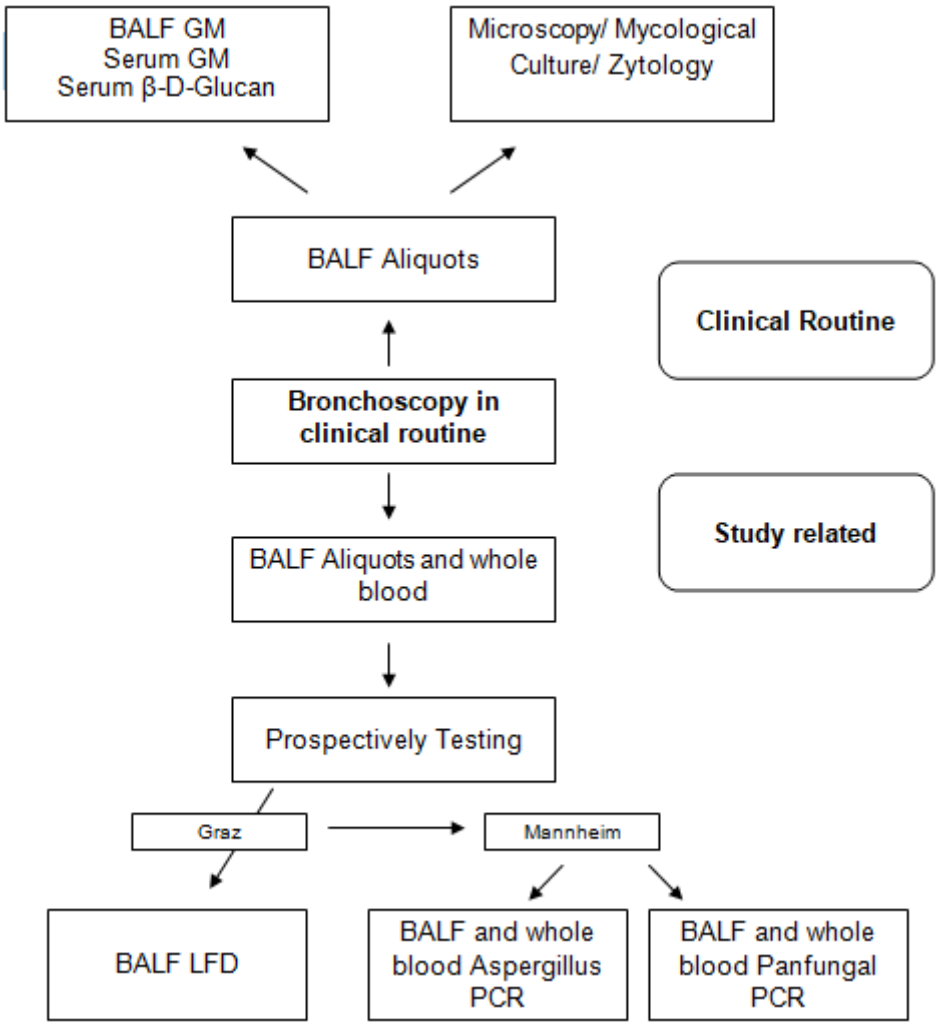


Figure 12: Routine and study-related procedures.

8.3.3 Galactomannan measuring

GM measurement was conducted initially at the Institute of Hygiene Microbiology and Environmental Medicine until October 2014. Afterwards the measurement of GM index was transferred to the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Austria, and to the Institute of Medical Microbiology and Hygiene of the University Hospital of Mannheim. GM concentrations were investigated by the Platelia *Aspergillus* enzyme immunoassay (Bio-Rad, Vienna, Austria) test kits in accordance with the manufacturer's instructions. The test variable was the titre of the *Aspergillus* GM antigen. Results were observed as an index relative to the optical density of the control sample obtained in the same run, with an index ≥ 0.5 considered positive in

case of serum and an index > 1.0 considered positive in case of BALF. GM testing was carried out routinely in serum and BALF (175). The final steps of GM measurement are displayed in figure 13 (positive samples in yellow) and figure 14 (optical density analysis).

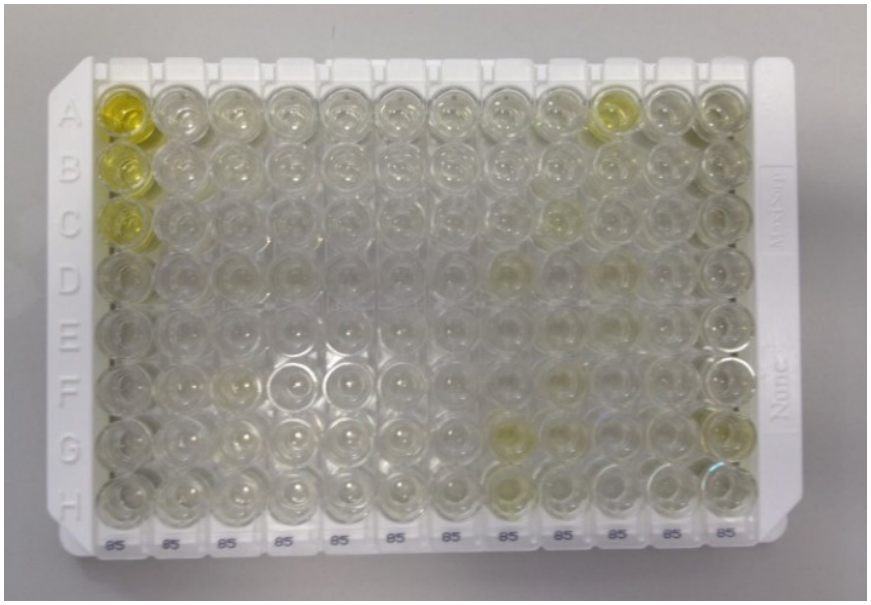


Figure 13: 96-well microplate with positive samples in yellow, by Frederike Reischies. Reproduced with permission. No changes were made.

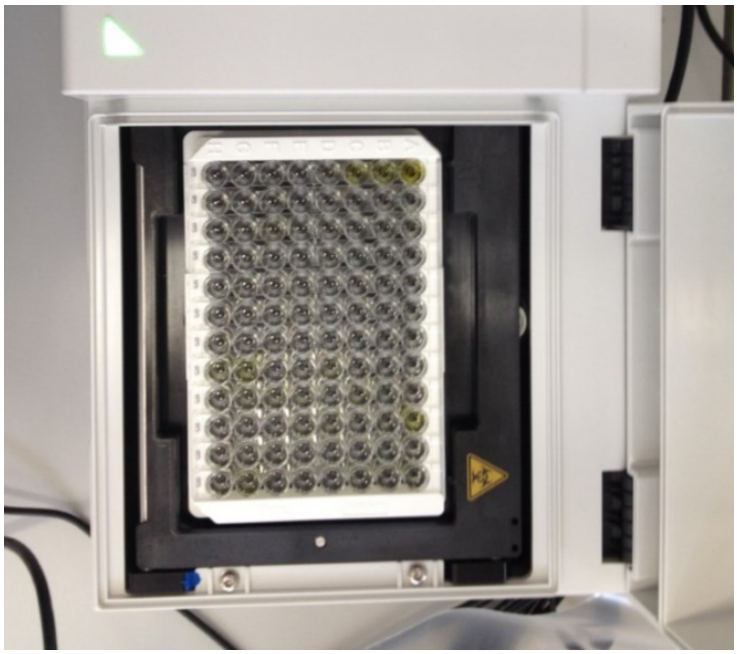


Figure 14: the 96-well microplate in the optical density reader. by Frederike Reischies. Reproduced with permission. No changes were made.

8.3.4 *Aspergillus* PCR and Panfungal PCR testing

In total 2 ml BALF/pleural effusion aliquot, biopsy specimens as well as 4 ml of whole blood were sent to the Scientific Lab of the University Hospital of Mannheim by using I-log for overnight shipping. A nested *Aspergillus* PCR assay was performed prospectively at the Scientific Lab of the University Hospital of Mannheim, Germany, according to the protocol of Skladny et al. (176, 179). Extraction of DNA was processed via phenol-chloroform extraction according to the protocol of Sambrook et al (180). As an internal control, a 138-bp PCR fragment encoded by the human glucose-6-phosphate dehydrogenase gene was amplified in each clinical sample (175).

8.4 Secondary analyses on BALF LFD performance

8.4.1 Performance of LFD in immunocompromised ICU patients

This multicentre cohort study was conducted at the three Austrian Medical University Hospitals of Graz, Innsbruck and Vienna, and at the University Hospital of Mannheim, Germany. Previously unpublished 149 BALF samples of 133 severely immunocompromised ICU patients at risk for invasive mould infection (IMI), which were routinely tested for the presence of *Aspergillus* species between January 2010 and June 2014, were included into this analysis. Patients with solid organ transplantation (SOT) were excluded. All specimens at the Medical University Hospital of Graz, Austria (*n* = 70), the Medical University Hospital of Vienna, Austria (*n* = 18), and the University Hospital of Mannheim, Germany (*n* = 10), were collected prospectively between February 2012 and June 2014. Moreover, in Vienna only BALF samples with culture evidence of *Aspergillus* were included. Samples at the Medical University Hospital Innsbruck were collected, in part prospectively (*n* = 31) between January 2013 and June 2013, in another part tested retrospectively (*n* = 20), which included in the Innsbruck fungal infection biobank sample collection between 2010 and 2012. The patient classification occurred in accordance to the EORTC/MSG criteria, which were adjusted by “ICU stay above 4 days” as host criteria. The reason for widening the revised EORTC/MSG criteria is due to the fact that the host factors in the EORTC/MSG guidelines were originally defined for patients with haematologic malignancies and for severely immunocompromised patients, but they have not been evaluated in other non-neutropenic patients at risk for IMI who do not fulfil the classic host criteria. Lateral Flow Device (LFD) testing in BALF specimens was performed retrospectively in the Microbiology Laboratory of the Department of Internal Medicine, Medical University of Graz; the Institute of Hygiene and Microbiology of the Innsbruck Medical University; the Division of Clinical Microbiology of the Medical University of Vienna, Austria; and the scientific laboratory of the Department of Haematology and Oncology of Mannheim University Hospital, Germany, depending on where the patient was enrolled (178).

8.4.2 Influence of antifungal treatment on LFD performance

Another retrospective cohort study was performed at the Medical University Hospital of Graz, Austria, and the University Hospital of Mannheim, Germany. The aim of this analysis was to evaluate the influence of systemic mould-active treatment on the sensitivity of the LFD test in BALF for diagnosing IA. A total of 63 BALF specimens obtained from 60 patients (59 samples at the Medical University of Graz, Austria, 4 samples at the Mannheim University Hospital, Germany) were used for the final analysis. Some of these patients and specimens have been published previously in our working group (99, 125, 157, 181, 182). All patients which were diagnosed with probable or proven IA between February 2011 and December 2014 and had exact documentation about the applied AF treatment for at least seven days prior to bronchoscopy were included in this retrospective analysis. Hence charts from patients who fulfilled probable or proven IA criteria of revised EORTC/MSG criteria were re-reviewed regarding mould-active treatment at the time of bronchoscopy (defined as mould-active AF initiated at least 24 hours prior to bronchoscopy). Sample collection and processing were carried out prospectively in both centres. LFD was performed prospectively and always at the day of BALF recovery at the Microbiology Laboratory of the Department of Internal Medicine of the Medical University of Graz and the Scientific Laboratory of the Department of Haematology and Oncology at the Mannheim University Hospital, Germany (177).

8.4.3 LFD procedure

For the point-of-care test 100 µl of untreated BALF was applied to the LFD with no pre-treatment. Results were read by naked eye after 15 minutes and interpreted according to C. Thornton's instructions as negative (-), weakly positive (+), moderately positive (++) or strongly positive (+++) (98), as shown in figure 15. Bound antigen-antibody-gold complexes were recorded as a red line, with intensity being proportional to the antigen concentration. Independent of the test line intensity, all positive results suggest germination of spores and active growth of the pathogen fungi in the lungs and thus were interpreted as positive. These LFD results were compared to the always routinely and prospectively performed and were compared with routinely performed BALF GM measurement, direct microscopy and conventional culture results (177, 178).

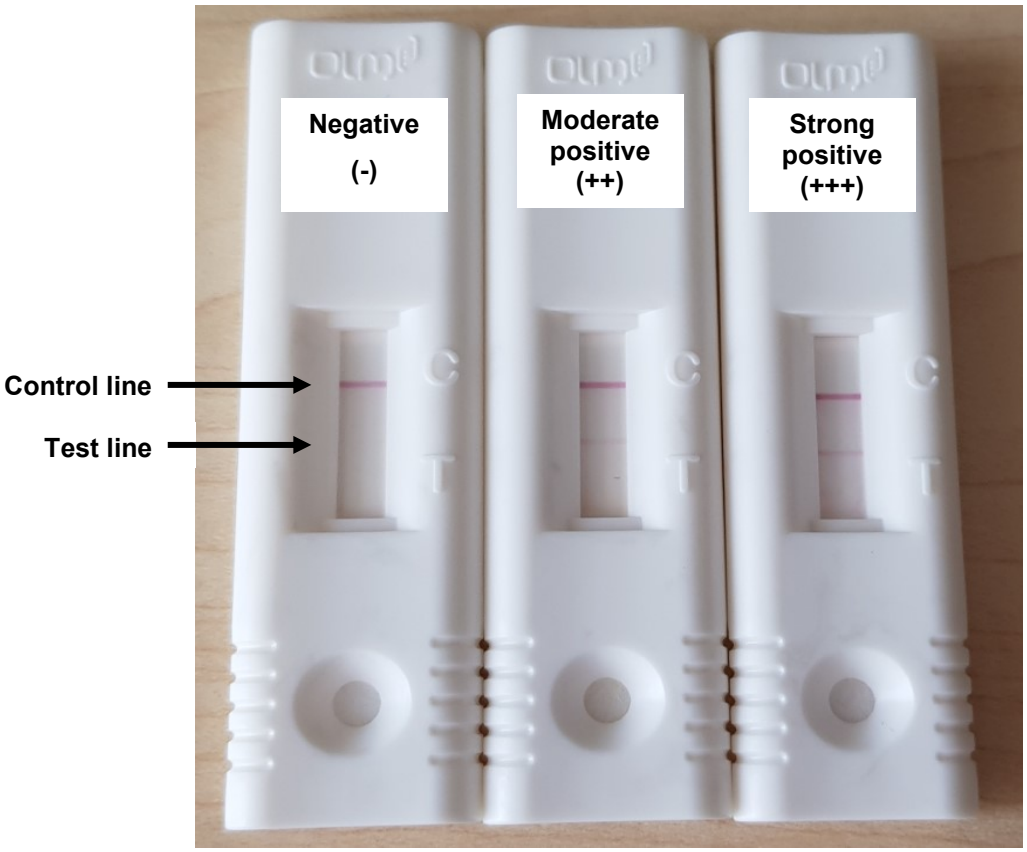


Figure 15: Lateral Flow Device test. Bound antigen-antibody complexes were recorded as a red line.

8.5 Statistical analysis

8.5.1 Primary analysis

Data collection and processing were done according to the Declaration of Helsinki, 1996, Good Clinical Practice, and the legal regulations and local ethics committee requirements. All data presented have been anonymised. The study protocol was approved by the local ethics committee of the Medical University Graz, Austria (EC-numbers 25–221 and 23–343) and the Mannheim University Hospital, Germany (EC-number 2012–320N-MA) and registered at ClinicalTrials.gov (Identifier: NCT02058316 and NCT01576653). Statistical analysis was performed using SPSS, version 22 (SPSS Inc., Chicago, IL USA). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for probable/proven IA versus no IA. Comparisons of the diagnostic test performances were made by using the diagnostic odds ratio (DOR) method. DOR values were described with 95% confidence intervals (95% CI). For the estimation of agreement between GM and PCR we used Cohen's kappa analysis (175).

8.5.2 Secondary analyses on BALF LFD performance

Secondary analyses were also conducted in accordance with the Declaration of Helsinki, 1996, Good Clinical Practice and applicable local regulatory requirements and laws. The study protocol was approved by the local ethics committee at the Medical University Graz, Austria (EC number 25-221 ex 12/13), as well as by the ethics committees of the Medical University of Vienna (EC number 1656/2013) and the Medical University of Innsbruck (EC number UN 4926), and the trials were registered at ClinicalTrials.gov (identifier NCT02058316). Patients treated at the Mannheim University Hospital were analysed retrospectively with a scientific intent, though approval by the local ethics committee (Faculty of Medicine in Mannheim Ethics Committee) was not required according to the German ethics committee regulations. All data have been anonymised and are not traceable to the individual patients. The ethics committees therefore did not require written informed consent of the enrolled participants. The performance analysis of a medical product was also announced to the Austrian Agency for Health and Food Safety (protocol number INS-621000-0478) (177, 178).

Statistical analysis was performed using SPSS version 22 software (SPSS Inc., Chicago, IL USA). The diagnostic performance of the LFD test in ICU patients for probable or proven IA versus no IA were analysed, and sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) were measured. Furthermore, diagnostic performance of BALF LFD, GM and conventional culture in patients with and without antifungal treatment were calculated. Sensitivities were compared using Fisher's exact test. Diagnostic odds ratio (DOR) values with 95% confidence intervals (95% CI) were defined, and p-values of < 0.05 were considered statistically significant (177, 178).

9 Results

9.1 Demographics

A total of 53 BALF and same-day blood samples of 53 patients were included in the final analysis. Thirty-eight patients (37 with haematological malignancies, 1 with active TB) undergoing bronchoscopy in daily routine and were prospectively enrolled at the Medical University of Graz, Austria. Fifteen patients with mixed underlying diseases (12 patients with haematological malignancy, 3 with other underlying diseases) with available same-day BALF and blood samples from July 2013 to May 2014 and suspected IA were enrolled retrospectively at the Mannheim University Hospital, Germany (175).

Of these 53 patients, 17 (32.1%) were male and 36 (67.9%) female, with a median age of 58 years. Underlying diseases of the study population were as follows: 17/53 (32.1%) acute myelogenous leukaemia (AML), 14/53 (26.4%) Non-Hodgkin lymphoma (NHL), 10/53 (18.8%) multiple myeloma (MM), 3/53 (5.7%) myelodysplastic syndrome (MDS), 2/53 (3.8%) chronic lymphocytic leukaemia (CLL), 2/53 (3.8%) acute lymphocytic leukaemia (ALL) and 5/53 (13.2%) other underlying disease (figure 16). Other underlying disease included cases of glioblastoma, active tuberculosis (IA), chronic myeloid leukaemia, primary myelofibrosis and bronchial carcinoma (no IA). Patients characteristics are illustrated in table 4 (175).

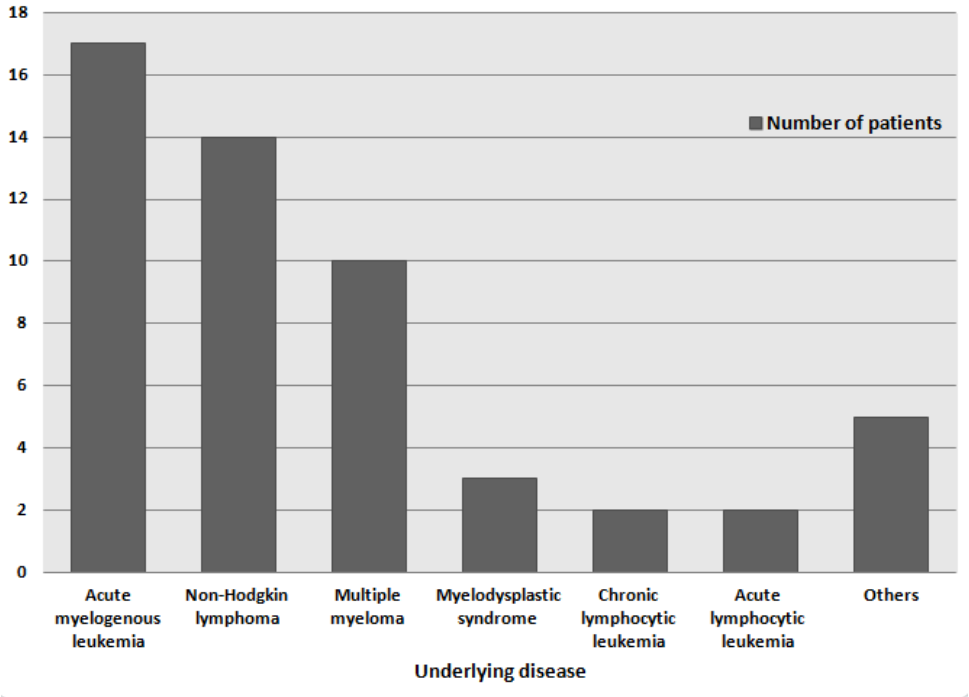


Figure 16: Distribution of underlying disease of the study cohort.

	Study cohort
Number of patients	53
Sex	
Male	17/53 (32.1%)
Female	36/53 (67.9%)
Median age (Range)	58 (26-83)
Underlying disease	
AML	17/53 (32.1%)
NHL	14/53 (26.4%)
MM	10/53 (18.8%)
MDS	3/53 (5.7%)
CLL	2/53 (3.8%)
ALL	2/53 (3.8%)
Others #	5/53 (13.2%)

cases of glioblastoma, active tuberculosis (IA), chronic myeloid leukaemia, primary myelofibrosis and bronchial carcinoma (no IA)

Table 4: Patients characteristics of the whole study cohort. Results reproduced from (175) with permission of Oxford University Press.

Of the whole study population more than half of patients, 34/53 (64.1%), received antifungal treatment before or during sample collection. 20/53 (35.8%) had no antifungal prophylaxis/therapy while study samples were obtained. Of the 16 cases with probable/proven IA 12 patients (75%) had received antifungal prophylaxis/therapy at the time of bronchoscopy and BALF recovery (median 2 days, IQR 1–10 days) (figure 17) (175).

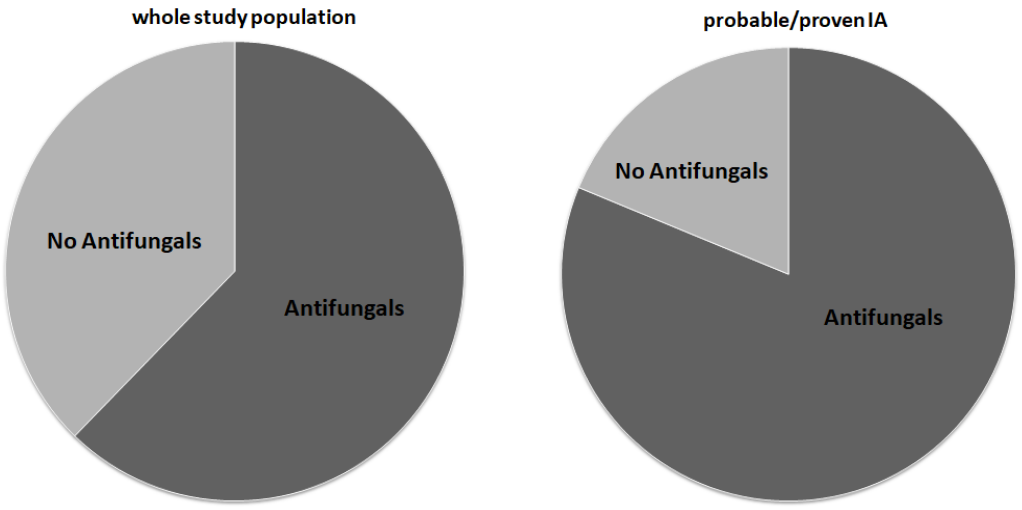


Figure 17: Distribution of antifungal treatment in the whole study cohort and in the probable/proven IA cases.

9.2 EORTC classification of the study cohort

Based on the 2008 revised EORTC/MSG criteria 2 (3.8%) patients were classified as proven and 14 (26.4%) as probable IA (altogether 16 patients, 30.2%), and 37 patients (69.8%) had no evidence for IA. Twenty-seven patients with possible IA (all negative *Aspergillus* PCR results in BALF and blood), and those who did not have GM and PCR results from BALF and same-day blood (*n* = 6) were excluded from the final analysis (figure 18) (175).

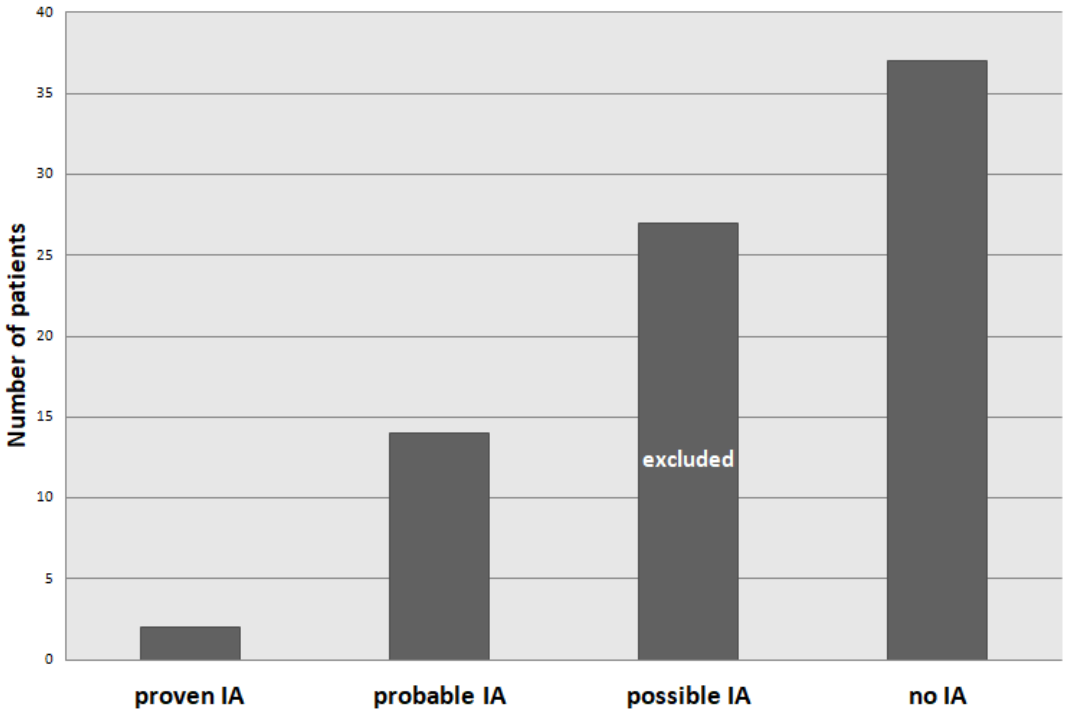


Figure 18: Classification of the study population according to the revised EORTC/MSG criteria.

Both proven cases were admitted at the University hospital of Graz, with underlying disease of acute myeloid leukaemia (AML) and active tuberculosis. Of the 14 probable cases, 6 (43%) were enrolled in Graz and 8 (53%) in Mannheim. Six (37.5%) were male and 10 (62.5%) were female, with a median age of 57 years (IQR: 46-83). These 16 patients were treated for the following diseases: 6/16 (37.5%) acute myelogenous leukaemia (AML), 3/16 (18.8%) Non-Hodgkin lymphoma (NHL), 4/16 (25%) multiple myeloma, 1/16 (6.2%) acute lymphocytic leukaemia (ALL) and 2/16 (18.8%) other underlying diseases with diminished immune response. Patients' characteristics of the probable/proven IA cases and those with no IA are shown in table 5 (175).

	probable/proven IA	no IA
Number of patients	16/53 (30.2%)	37/53 (69.8%)
Sex		
Male	6/16 (37.5%)	11/37 (29.7%)
Female	10/16 (62.5%)	26/37 (70.3%)
Median age (Range)	57 (46-83)	60 (26-82)
Underlying disease		
AML	6/16 (37.5%)	11/37 (29.7%)
NHL	3/16 (18.8%)	11/37 (29.7%)
MM	4/16 (25%)	6/37 (16.2%)
MDS	0/16	3/37 (8.1%)
CLL	0/16	2/37 (5.4%)
ALL	1/16 (6.2%)	1/37 (2.7%)
Others #	2/16 (18.8%%)	3/37 (10.8%)

cases of glioblastoma, active tuberculosis (IA), chronic myeloid leukaemia, primary myelofibrosis and bronchial carcinoma (no IA)

Table 5: Patients characteristics of the probable/proven IA and no IA cases. Results reproduced from (175) with permission of Oxford University Press.

9.3 Diagnostic Performance of PCR

The study related corresponding blood samples taken at a maximum of 24 hours after bronchoscopy and BALF recovery. *Aspergillus*-specific PCR and the panfungal PCR was conducted at the scientific lab of the Mannheim University as described before. Seven of the 16 (43.8%) classified as probable/proven IA patients showed a positive *Aspergillus*-specific PCR result out of BALF. All other 37 patients without IA had no evidence of *Aspergillus* in BALF PCR. The *Aspergillus* PCR out of the concurrent same-day blood samples resulted negative in all 53 patients, even in the 16 probable/proven cases. Specificity of PCR in BALF as well as in the corresponding blood samples was 100%. PPV and NPV of PCR in BALF were 100% and 80.4%, respectively (table 6) (175).

probable/proven vs. no IA		
PCR	**BALF**	**whole blood**
Sensitivity	7/16 (43.8%)	0/16
Specificity	37/37 (100%)	37/37 (100%)
PPV	7/7 (100%)	0/0
NPV	37/46 (80.4%)	37/37 (100%)
DOR	59.2 (3.1-1,1132)	NA

Table 6: Diagnostic performance of PCR. Results reproduced from (175) with permission of Oxford University Press.

9.4 Diagnostic Performance of GM

When using the cut-off 0.5 ODI for GM 6/16 patients with proven/probable IA were positive in BALF (38%) and 5/16 patients (31%) when elevating the cut-off on 1.0 ODI. Measurement of Galactomannan index in the corresponding same-day serum yielded positive in 5/16 patients (31%) with proven/probable IA (cut-off 0.5 ODI). Sensitivity, specificity, PPV and NPV of BALF GM with the cut off 0.5 ODI were 37.5%, 91.9%, 66.7% and 77.3%, respectively. Using the cut-off 1.0 ODI specificity of BALF GM increased (94.6%), indeed sensitivity were lowered (31.3%). Diagnostic performance of serum GM and BALF GM using the cut-off 0.5 ODI and 1.0 ODI are displayed in table 7 (175).

	probable/proven vs. no IA		
GM	**BALF 0.5 ODI**	**BALF 1.0 ODI**	**Serum 0.5 ODI**
Sensitivity	6/16 (37.5%)	5/16 (31.3%)	5/16 (31.3%)
Specificity	34/37 (91.9%)	35/37 (94.6%)	37/37 (100%)
PPV	6/9 (66.7%)	5/7 (71.4%)	5/5 (100%)
NPV	34/44 (77.3%)	35/46 (76.1%)	37/48 (77.1%)
DOR	6.8 (1.4-32.2)	8.0 (1.3-46.9)	35.9 (1.8-699.3)

Table 7: Diagnostic performance of GM. Results reproduced from (175) with permission of Oxford University Press.

9.5 Test combination

Cohen's kappa analysis used for measuring the agreement between the *Aspergillus* specific PCR in BALF and BALF GM index, resulted in a value of 0.18 [−0.16 to 0.52] with the strength of agreement to be regarded as poor (κ < 0.2). Sensitivities of BALF PCR testing and BALF GM testing with cut off 1.0 ODI as single test were 43.8% and 31.3%, respectively. Both diagnostic tests in BALF combined lead to an increased sensitivity of 62.5% with a still high specificity of 95%. Moreover, the addition of the serum GM testing raised the sensitivity up to 68.8%, while the specificity did not shown any difference (94.6%). Finally, the combination of BALF PCR, GM and culture as well as concurrent same-day serum GM increased the sensitivity further to 75.0%, with the unaltered specificity of 95%, independent of the different combinations of the diagnostic tests (PPV 85.7%, NPV 89.7%). An overview of the diagnostic performance of *Aspergillus*-specific PCR, GM and conventional culture in lavage fluid and whole blood/serum samples as single tests or in combinations is shown in table 8 and 9 (175).

After GM exclusion as mycological evidence 12 (86%) of the 14 probable IA cases still met the definition. Thus, of these two patients probable IA was met by positive GM as mycological evidence alone. Sensitivities without these two cases for probable/proven IA (*n* = 14) were 50% for *Aspergillus* specific PCR in BALF, 0% for *Aspergillus* specific PCR in same-day blood, 36% for BALF-GM with cut-off 0.5 ODI and 29% with cut-off 1.0 ODI and 21% for GM in same-day serum sample (175).

probable/proven vs. no IA		
Diagnostic test	**Sensitivity**	**Specificity**
Culture BALF	3/16 (18.8%)	37/37 (100%)
GM BALF 1.0 ODI and/or GM serum 0.5 ODI	7/16 (43.8%)	35/37 (94.6%)
PCR BALF and/or GM serum 0.5 ODI	10/16 (62.5%)	37/37 (100%)
PCR BALF and/or GM BALF 1.0 ODI	10/16 (62.5%)	35/37 (94.6%)
PCR BALF and/or GM BALF 1.0 ODI and/or GM serum 0.5 ODI	11/16 (68.8%)	35/37 (94.6%)
PCR BALF and/or GM BALF 1.0 ODI and/or BALF culture and/or GM serum 0.5 ODI	12/16 (75.0%)	35/37 (94.6%)

Table 8: Sensitivity and specificity of PCR, GM and culture in BAF, and in whole blood/serum samples as single test and in combination. Results reproduced from (175) with permission of Oxford University Press.

probable/proven vs. no IA			
Diagnostic test	**PPV**	**NPV**	**DOR**
PCR			
BALF	7/7 (100%)	37/46 (80.4%)	59.2 (3.1-1.1132)
Whole blood	0/0	37/37 (100%)	NA
GM			
BALF 0.5 ODI	6/9 (66.7%)	34/44 (77.3%)	6.8 (1.4-32.2)
Serum 0.5 ODI	5/5 (100%)	37/48 (77.1%)	35.9 (1.8-699.3)
BALF 1.0 ODI	5/7 (71.4%)	35/46 (76.1%)	8.0 (1.3-46.9)
Culture BALF	3/3 (100%)	37/50 (76.1%)	19.44 (0.9-401.9)
GM BALF 1.0 ODI and/or GM serum 0.5 ODI	7/9 (77.8%)	35/46 (79.6%)	13.6 (2.4-77.1)
PCR BALF and/or GM serum 0.5 ODI	10/10 (100%)	37/43 (86.1%)	121.2 (6.3-2332)
PCR BALF and/or GM BALF 1.0 ODI	10/12 (83.3%)	35/41 (85.4%)	29.2 (5.1-167.5)
PCR BALF and/or GM BALF 1.0 ODI and/or GM serum 0.5 ODI	11/13 (84.6%)	35/40 (87.5%)	38.5 (6.5-227.1)
PCR BALF and/or GM BALF 1.0 ODI and/or BALF culture and/or GM serum 0.5 ODI	12/14 (85.7%)	35/39 (89.7%)	52.5 (8.5-324.0)

Table 9: PPV, NPV and DOR of PCR, GM and culture in BALF, and in whole blood/serum samples as single test and in combination. Results reproduced from (175) with permission of Oxford University Press.

9.6 Results of the secondary analyses on BALF LFD performance

9.6.1 Performance of LFD in immunocompromised ICU patients

Ultimately 133 patients were included in the analysis, all treated in an intensive care unit stetting at the time of bronchoscopy and BALF recovery. Of these 133 patients, 149 BALF specimens were collected and analysed. The median age of the study cohort was 60 years. Eighty-seven (65.4%) were male and 46 (34.6%) were female. The main underlying risk factors for invasive pulmonary aspergillosis of the study population were, among others, COPD (21.5%), acute leukaemia (12%), neutropenia (12%), chronic systemic corticosteroid administration (7%), bone marrow transplantation (6%), lung cancer (5%), influenza A viral pneumonia (5%) and liver cirrhosis/alcoholic hepatitis (4%) (178).

According to the 2008 revised EORTC/MSG criteria 2/133 patients were classified as proven and 14/133 patients as probable IA, altogether 16/133 (12% of patients, 20/149 BALF samples). Both proven IA cases died during the ICU stay, with underlying diseases of acute myelogenous leukaemia and acute on chronic liver failure. Underlying diseases of the 14 probable IA cases were as follows: COPD (4/14, 28.6%), sepsis due to pneumonia (4/14, 28.6%) including two influenza A viral pneumonia, hematologic malignancies (3/14, 21.4%), liver cirrhosis/alcoholic hepatitis (2/14, 14.3%) and trauma (1/14, 7.1%). The majority of probable IA cases (9/14, 64.3%; 11/16 BALF samples) did fulfil EORTC/MSG host criteria, while 5 patients were classified as probable IA (35.7%; 11/16 BALF samples) because of fulfilling the newly introduced host criteria of ICU stay above 4 days (all had a minimum ICU stay of 10 days). A GM immunoassay was conducted in 10/16 BALF samples of probable IA cases. Six BALF samples were not tested. Out of the ten tested, 9 BALF samples resulted GM positive. Two of these had additional positive *Aspergillus* culture. The remaining seven BALF samples of patients with probable IA yielded a positive *Aspergillus* culture (178).

When we used the modified EORTC/MSG criteria, sensitivity and specificity of the BALF LFD test for probable/proven IA in our study cohort were 80% and 81%, respectively. When we used the predefined EORTC/MSG criteria, sensitivity and specificity were 87% and 81%, respectively. An overview of the diagnostic performance of the LFD for probable/proven IA versus no IA is depicted in table 10 and 11. In the GM-positive BALF samples, the sensitivity of LFD was 89% (8/9 resulted LFD positive). The sensitivity in the

BALF samples of patients with probable IA with positive *Aspergillus* culture but missing GM measurement was 75% (6/8 resulted LFD positive) (178).

LFD test yielded positive in 20/108 (19%) BALF samples that did not fulfil modified EORTC/MSG criteria. Six of these 20 BALF samples had mycological evidence for aspergillosis (all were *Aspergillus* culture positive, and one showed additionally a high BALF GM level over 5 ODI and a positive serum GM), but the patients did not fulfil clinical criteria for IA. The majority of the false positive LFD results yielded only weak positives. Four of 20 BALF samples (20%) of four patients with probable/proven IA were falsely negative (1/4 BALF samples of a patient with proven IA, which turned negative under antimould-active-therapy). Three of the four false negative BALF LFD tests showed positive *Aspergillus* culture and 1 was BALF GM positive (ODI = 0.72). BALF GM level measuring was performed in 53/149 BALF samples. Twelve of 53 (22.6%) were positive (ODI > 0.5). Positive BALF GM levels were seen in 9/10 patients with probable/proven IA (178).

probable/proven vs. no IA		
LFD test performance	**Sensitivity**	**Specificity**
Modified EORTC/MSG criteria		
Overall study population	16/20 (80%)	88/108 (81%)
Graz	5/6 (83%)	46/58 (79%)
Innsbruck	6/8 (75%)	35/37 (95%)
Vienna	4/5 (80%)	7/13 (54%)
Mannheim	1/1 (100%)	7/9 (78%)
Original revised EORTC/MSG criteria		
Overall study population	13/15 (87%)	101/124 (81%)
Algorithm according to Blot et al.		
Overall study population	10/12 (83%)	108/137 (79%)

Table 10: Sensitivity and specificity of the LFD test for probable/proven IA versus no IA using three different classification criteria: modified EORTC/MSG criteria (including intensive care unit stay over 4 days as host criteria), original 2008 revised EORTC/MSG criteria (69) and the clinical algorithm according to Blot et al. (183). Results reproduced from (178), published open access.

probable/proven vs. no IA			
LFD test performance	**PPV**	**NPV**	**DOR**
Modified EORTC/MSG criteria			
Overall study population	16/36 (44%)	88/92 (96%)	17.6 (5.3-58.3)
Graz	5/17 (29%)	46/47 (98%)	19.2 (2-179.9)
Innsbruck	6/8 (75%)	35/37 (95%)	52.5 (6.2-447.6)
Vienna	4/10 (40%)	7/8 (88%)	4.7 (0.4-54)
Mannheim	1/3 (33%)	7/7 (100%)	9 (0.3-200)
Original revised EORTC/MSG criteria			
Overall study population	13/36 (36%)	101/103 (98%)	28.5 (6-135)
Algorithm according to Blot et al.			
Overall study population	10/39 (26%)	108/110 (98%)	18.6 (3.9-89.7)

Table 11: PPV, NPV and DOR of the LFD test for probable/proven IA versus no IA using three different classification criteria: modified EORTC/MSG criteria (including intensive care unit stay over 4 days as host criteria), original 2008 revised EORTC/MSG criteria (69) and the clinical algorithm according to Blot et al. (183). Results reproduced from (178), published open access.

9.6.2 Influence of antifungal treatment on LFD performance

In total 63 BALF samples of 60 participants were included in the final analysis. All patients were classified as having probable (54/60, 57 BALF samples) or proven IA (6/60, 6 BALF samples). The majority of the BALF samples (59/63) were obtained in Graz, Austria. The rest (4/63) were obtained in Mannheim, Germany. Half of the BALF samples from Mannheim were obtained from patients classified as proven IA. The underlying diseases of the patients from Mannheim were haematological malignancy (3/4, 75%) and central nervous system malignancy (1/4, 25%). Overall 19/60 (31.6%) patients (21/63 BALF samples) received mould-active antifungal treatment prior to bronchoscopy and BALF recovery (177).

The overall sensitivity of the BALF LFD test for probable/proven IA was 75%. Sensitivity in patients with antifungal treatment initiation at the time of BALF sampling was significantly lower (52%) in comparison to those without antifungal agents (86%, $P = 0.006$). In the whole study cohort 16/63 (25.4%) BALF samples had a false negative LFD test result. Ten of those 16 BALF samples were gathered from patients who received systemic anti-mould-active treatment prior to LFD testing, two patients for prophylaxis and eight had mould active antifungal treatment. In two of these BALF samples GM and *Aspergillus* culture also yielded negative, and mycological criteria was fulfilled by positive serum GM alone. 11/21 BALF samples depicted true positive LFD test results despite ongoing antifungal treatment. The duration of mould-active treatment in true-positive results (median: 12 days; range: 1–49 days) did not differ considerably compared to those with false-negative LFD results (median: 15 days; range: 2–150 days) (177).

The sensitivity of BALF GM was also reduced in those with antifungal (AF) treatment when using the cut-off 0.5 ODI (71% under AF versus 95% without) and decreased significantly more when using the cut-off 1.0 ODI (52% under AF versus 81% without). In our cohort antifungal treatment had no significant impact on the sensitivity of BALF culture, but it has to be emphasized that the sensitivity of the conventional culture was low in general (25%). Overview of sensitivities of BALF LFD test, BALF GM and BALF culture for probable/proven IA in patients with and without antifungal prophylaxis/therapy are displayed in table 12 and 13 (177).

BALF LFD sensitivity			
	Overall	**in probable IA cases**	**in proven IA cases**
Overall study population	47/63 (75%)	42/57 (74%)	5/6 (83%)
Under systemic AFs	11/21 (52%)	10/20 (50%)	1/1 (100%)
Voriconazole	4/10 (40%)	4/10 (40%)	-
Posaconazole	3/4 (75%)	2/3 (67%)	1/1 (100%)
Caspofungin	3/5 (60%)	3/5 (60%)	-
liposomal AmB	2/3 (67%)	2/3 (67%)	-
Without AFs	36/42 (86%)	32/37 (86%)	4/5 (80%)

Table 12: Sensitivities of BALF LFD for probable/proven IA in patients with and without antifungal (AF) prophylaxis/therapy. Results reproduced from (177) with permission of Elsevier.

	BALF GM sensitivity		**Culture sensitivity**
	Cut-off 0.5 ODI	**Cut-off 1.0 ODI**	
Overall study population	55/63 (87%)	45/63 (71%)	16/63 (25%)
Under systemic AFs	15/21 (71%)	11/21 (52%)	3/21 (14%)
Voriconazole	9/10 (90%)	7/10 (70%)	1/10 (10%)
Posaconazole	0/4 (0%)	0/4 (0%)	1/4 (25%)
Caspofungin	4/5 (80%)	3/5 (60%)	1/5 (20%)
liposomal AmB	2/3 (67%)	1/3 (33%)	0%
Without AFs	40/42 (95%)	34/42 (81%)	13/42 (31%)

Table 13: Sensitivities of BALF GM and BALF culture for probable/proven IA in patients with and without antifungal (AF) prophylaxis/therapy. Results reproduced from (177) with permission of Elsevier.

10 Discussion

Diagnosing life-threatening invasive mould infections in immunocompromised patients is still challenging. Early detection is crucial for optimal therapeutic success and overall survival. In the last years diagnostic tests were amplified and constantly improved. Nevertheless, the optimal use of the available diagnostic tools is still unclear (175).

In this study we evaluated a cohort of immunosuppressed patients for whom BALF and serum samples were collected within 24 hours of one another for GM and *Aspergillus*-specific PCR testing. Our main finding was that the sensitivity was low in every diagnostic test when interpreted on their own, regardless of which body fluid was used. When using blood specimens, it was even lower than in BALF samples (175).

In a previous study by Lass-Flörl et al., the sensitivity of PCR in BALF and whole blood samples were 100% and 40%, respectively, for patients with proven infection. *Aspergillus*-specific PCR performance in blood is in addition more affected by ongoing antifungal treatment (184). Presumably, antifungal therapy effectuates the clearance of *Aspergillus* in blood at least to undetectable levels, whereas this clearance effect does not occur in lung tissue specimens. In another prospective two-year study, positive *Aspergillus* PCR results turned negative directly after antimycotic treatment start, although this did not correlate with the clinical condition after treatment initiation in these patients (185). Responsible for that could be the short plasma half-life of DNA, which is supposed to be maximum 4 minutes (186). Another reason for that could be the need of a certain fungal load for positive PCR test results in blood. Löffler et al. verified a correlation between a high fungal burden in tissue and the concomitant circulating fungal DNA in blood. Consequently, PCR detection of fungal DNA in blood may indicate a high fungal load and the severity of disease, and negative PCR blood results do not exclude the possibility of the presence of invasive aspergillosis. In their study, Löffler et al. also illustrated a higher sensitivity of PCR in whole blood samples than in plasma samples, while additionally noting that DNA extraction from plasma was more challenging (187).

Our results depicted a significantly better diagnostic performance of *Aspergillus* PCR in BALF compared to blood when used as single test performed in patients with diminished immune reaction. In this study blood PCR did not result positive in a single probable or proven IA case. This might be explained by the diminished fungal DNA burden in the blood in cases of prior antifungal therapy or prophylaxis. Another explanation might be

that we used a one-time PCR test in contrast to a serial PCR screening. In turn, the BALF PCR results were demonstrated to be superior among all diagnostic tests and samples: sensitivity and specificity were 43.8% and 100%, respectively (175).

When using GM index levels for diagnosis of IA, serum GM was also less sensitive than BALF GM. In 2015 Boch et al. published a retrospective analysis of the diagnostic performance of BALF GM and concomitant serum GM of the same immunocompromised patients at the same time point of infection, in a time frame of 24 hours prior to or post bronchoscopy. Sensitivity and specificity were 85% and 88% for BALF versus 23% and 88% for serum (188). Poor diagnostic performance of GM in serum was also reported in other studies, particularly in the presence of antifungal prophylaxis (135, 189). Sensitivity of GM seems to be higher the closer the serum specimens were collected to the time of diagnosis, because the day of diagnosis of suspected IA is supposed to be the day of highest fungal load. Patients receiving preventive antifungal therapy showed a low quantity of circulating GM in the blood stream, hence it is to be assumed that mould-active prophylaxis in patients at high risk for IA decreases fungal burden and GM index values (135). In addition, different antifungal agents probably influence GM index levels differently. Echinocandins paradoxically raise circulating serum GM levels in experimental pulmonary aspergillosis in neutropenic rabbits by inducing hyphal damage, despite significantly improved survival (190). The interpretation of serum GM levels should consider the type, the duration and the dose of ongoing antifungal treatment.

The measurement of the serum GM index is potentially also of limited value in patients with a normal neutrophil count. Neutrophils are responsible for destroying the germinating conidia and for the prevention of *Aspergillus* angioinvasion by the secretion of fungicidal proteins and reactive oxygen species. Consequently, if the cellular immunity is undamaged, angioinvasion is very limited and the level of circulating GM will be lowered. When using GM as a biomarker for screening in blood specimens, the Platelia ELISA may show false negative results specifically in non-neutropenic patients, where IA tends to grow airway-invasive only (191, 192).

BALF GM and serum GM results in our patient population did not differ markedly in terms of sensitivity: 31% sensitivity for serum versus 38% for BALF when using the cut-off 0.5 ODI. The combination of BALF GM and serum GM measurement led to an increased sensitivity of up to 43.8% (175). Under antifungal treatment the diagnostic performance of GM was lower, but at the same level like in other studies with a higher percentage of included patients under ongoing mould-active treatment prior to mycological workup (193).

Besides performance of all available diagnostic tests are impacted by antifungal treatment as well (79).

Previous studies illustrated the clinical significance of GM and also PCR testing for diagnosis of IA in various patient cohorts (194-197). Conventional culture, historically used to define proven cases of IA, is limited by long turnaround time and lack of sensitivity (113), which is also shown in this study (sensitivity 18.8%) (175). This depicts the substantial importance of biomarker testing in the daily routine of IA diagnosis in patients at risk for IA. However, conventional culture is highly specific (100% in this study) and still remains essential for detecting other moulds, like Mucorales, that are not detectable by GM immunoassay. Moreover, BALF culture is particularly fundamental for broad susceptibility and resistance testing (175). In the last years an increased number of azole-resistant (itraconazole, voriconazole, posaconazole) invasive aspergillosis has occurred with high mortality rates among these patients (128, 198, 199). So far, the detection of azole-resistance-mediating cyp51A gene mutations was only based on a positive culture result. Meanwhile, PCR assays, for the detection of azole resistance in *Aspergillus fumigatus* causing invasive aspergillosis, were developed and seem to be very promising in the management of mould-active treatment (200, 201). Nevertheless, conventional culture nowadays is still indispensable due to the fact of the increasing incidence of the cyp51 key mutation in *Aspergillus* (202). Vermeulen et al. illustrated in 2013 that the azole resistance in *Aspergillus spp.* is evolving into a threaten global health problem (203).

In the present study we demonstrated that the combination of *Aspergillus* DNA detection by PCR with concurrent biomarker detection of fungal cell wall components leads to a noticeably increased sensitivity (175). The benefit of this diagnostic tool combination was also shown in another study, which investigated the diagnostic performance of GM and PCR: the combination of BALF GM and PCR achieved 100% sensitivity (157). Besides, the performance of both diagnostic procedures seems to be better in BALF specimens, hence from the site of infection, than in peripheral blood samples (157, 175, 204).

However, the ability to compare results between studies is often complicated by type and duration of mould-active prophylaxis/therapy or other antimicrobial medication, the underlying disease, or the grade of immunodeficiency. All of these factors do not only influence the development of an invasive fungal infection, but they also have an impact on diagnostic test performance. Synchronising those influences could provide a higher significance of the diagnostic potential of BALF as sampled from the direct site of infection as well as of the simultaneous serum samples rapidly available in clinical routine. This

represents the first study to evaluate blood specimens which were collected within a maximum of 24 hours of BALF recovery, often directly after or before the intervention. Thus, the substantial concept of this study was that the precondition of and the influences on the BALF specimens and concurrent blood samples in patients with immune deficiency at risk for IA were the same, to identify the ideal type of sample for diagnostic test performance when obtained under the same conditions (like antifungal treatment) (175).

Morrissey et al. postulated recently that the combination of serum GM and whole blood PCR testing is effective for directing the use of antifungal therapy for invasive aspergillosis and in reducing the use of empirical anti-mould-active treatment in patients with haematological malignancies (81). In contrast to numerous other studies, in a multicentre retrospective study in Australia a decreased sensitivity of BALF GM and PCR in the presence of antifungal treatment was not seen; instead a small effect on the specificity of BALF GM was found, caused by β-lactam antibiotics (204). Indeed, Mikulska et al. published two years prior that piperacillin/tazobactam seems to be no longer responsible for false-positive GM results nowadays, although some residual GM might still be present in piperacillin/tazobactam (132).

GM detection is the current gold standard in diagnosis of IA and defined as mycological criterion for probable IA in the 2008 revised EORTC/MSG criteria. In this study we focused on the ideal test combination for an improved diagnostic performance, with the challenge of determining the overall performance of a biomarker test, which is already used as a standard diagnostic tool (175). Recommendations for the ideal BALF GM cut-off vary widely, between 0.5 and 3.0 optical density index (148, 196, 205, 206). In the Guidance on Qualification of Biomarkers, the FDA recently elevated the GM cut-off in BALF specimens from 0.5 to 1.0 ODI, while GM still has the same cut-off in the EORTC/MSG criteria (207). We evaluated the lower 0.5 cut-off as well as the higher 1.0 cut-off. Not surprisingly, the specificity of BALF GM increased when using the higher cut-off (from 91.9% up to 94.6%) but concurrently sensitivity decreased (from 37.5% down to 31.3%) (175).

While the optimal cut-off of GM assay is still a matter of debate, PCR is not yet implemented in the EORTC/MSG criteria. A rationale not to include the PCR assay in a revised version is the absence of test standardization. We used a well-evaluated nested *Aspergillus*-specific PCR protocol, which demonstrated its performance in diverse and in comparable multicentre studies mainly among centres in Austria and Germany (164, 165, 169, 178, 194). Nevertheless, it has to be emphasized that our data cannot be transferred

to other PCR methods, as performance of currently used PCR assays differ widely due to lacking external standardization. Another limitation of PCR testing in general is the need of an appropriate quantity of material (minimum 2 ml) for valid testing, in comparison to GM index measurement, for which markedly less amount of specimen (600 µl) is required for test performance (175).

Knowing the advantages and disadvantages of every diagnostic tool is essential for timely diagnosis, as well as the type of sample used. Our results confirm the diagnostic strategy of obtaining specimens from the direct site of infection that applies in cases of IA, bronchoscopy and BALF recovery. However, the process of BALF-sampling can be biased by the examiner (175). Theoretically a standardized procedure for recovery of lavage fluid exists, though in clinical routine there is often a wide diversity in procedures, particularly concerning the volume of BALF. The diverse quantity of used saline and the recovered volume of fluid for the BALF procedure varied individually and across the different centres, which may impair GM and DNA concentration and consequently the sensitivity and cut-off value for BALF GM assay. Racil et al. published a trend towards a higher amount of aspirated fluid in negative BALF GM results, whereas the volume of instilled fluid seems to have no influence in positive GM testing (208).

In addition, BALF pre-treatment procedures prior to GM index measurement were also shown to have an impact on GM levels (182). Mucolytic pre-treatment with dithiothreitol of viscous respiratory specimens is very effective in liquefying and often used for viscous sputum samples of cystic fibrosis patients (209, 210). In 2014 the use of dithiothreitol as liquidation agent was recommended in the Aspergillus Guidelines of the European Society for Clinical Microbiology and Infectious Diseases (211). A commercially available dithiothreitol is Sputasol®, an Oxiod Microbiological Product, which is often used in microbiologic laboratories. Main principal in mucolytic pre-treatment and in decreasing viscosity of a respiratory sample is to improve the homogenization of the sample and the equal distribution of the pathogens in the sample. In a previous study significantly lower GM levels in BALF specimens pre-treated with Sputasol® were observed and were verified by retrospectively tested frozen samples (182). No mucolytic agents were used in samples of our study prior to GM measurement.

While performance of diagnostic tests for IA is superior in BALF, the risk of severe complication, e.g. bleeding in case of thrombocytopenia or acute respiratory distress syndrome, often results in a delay or even in avoiding the essential diagnostic bronchoscopy in heavily immunosuppressed patients. A prospective multicentre analysis

illustrated a clear benefit and a small complication rate of invasive diagnostic procedures like bronchoscopy with BALF recovery in immunocompromised patients with lung infiltrates and acute respiratory failure (212). Other studies have also reported a significant diagnostic gain which outweighs the risk of procedure-related complication (106, 157).

While agreement analyses showed a poor strength of agreement (κ = 0.18) between PCR and GM, combined BALF PCR and BALF GM (i.e. at least one positive) achieved arising increasing combined sensitivity with a still high specificity over 90%. If further combined with serum GM, the diagnostic test performance increases even more (sensitivity 68.8%, DOR 38.5 [6.5– 227.1]). Combination of all diagnostic tools (conventional culture, PCR, GM) in BALF together with serum GM resulted in the highest overall diagnostic test performance (175).

One limitation of this study was the small number of proven IA cases, like in several publications evaluating the diagnostic performance of biomarker tests and the potential influence of antifungal treatment for the diagnosis of invasive aspergillosis (79, 99, 157, 177, 213). It is challenging to obtain a sufficient number of probable/proven cases for the calculation of the diagnostic potential of a test in a prospective single centre study alone. Consequently, we decided to expand our cohort by including cases from Mannheim, Germany, which were included retrospectively (175).

In addition, for better interpretation of the results, we evaluated BALF LFD performance in a subset of the prospectively enrolled cases with haematological malignancies, performed an analysis of BAF LFD performance in a mixed cohort of immunocompromised patients and evaluated the influence of mould-active antifungal treatment on the performance of the point-of-care test in patients with invasive pulmonary aspergillosis (177, 178).

ICU patients are highly susceptible to invasive fungal infections; incidence rates in Europe are increasing in the last decades and range up to 7% (71, 214-216). In our multicentre analysis of four participating centres, the prevalence in the mixed cohort of ICU patients was even higher (11%). We only included critically ill patients, who underwent routine bronchoscopy and routine mycological workup of the BALF ordered by the treating physicians autonomously. Thus, the whole study population was at high risk for probable/proven IA, which may be the reason for the high prevalence rate (178). In numerous hospitals, GM immunoassay testing is not available on the same day, which could lead to a fatal delay in treatment initiation. To overcome this problem, the LFD test was developed, and its high diagnostic potential in BALF and serum samples of non-ICU patients has been illustrated in various studies (97-99, 160, 181, 217). Especially notable

were the high negative predictive values for ruling out invasive pulmonary aspergillosis (125, 157, 181).

Willinger et al. demonstrated a sensitivity and specificity of BALF LFD test in solid organ transplant patients of 91% and 83%, respectively (125). These results are comparable and almost the same as those of BALF GM in SOT-patients, which displayed a sensitivity of 93% and specificity of 95% in lung transplant recipients (218). In a mixed cohort of patients with diminished immune response, a sensitivity of 80% and a specificity of 95% of BALF LFD test was published. The combination of a positive LFD and positive GM results seems especially to be very promising for the diagnosis of IA in high-risk patients: the sensitivity increased up to 90% with a still high specificity of 93% (157). In a study population with underlying respiratory diseases, sensitivity and specificity of the LFD test were 77% and 90%, respectively. In this study 104 of 226 immunocompromised patients were treated in an ICU setting (99), which underscores that IA is an increasing problem and dreaded threat among ICU patients nowadays.

Azoulay et al. reported a remarkable overtreatment of critically ill patients in a large multicentre study. Two thirds of ICU patients with antifungal treatment had received antifungal agents without documented invasive fungal infection (219). This could be compensated by the rapidly available LFD test in BALF specimens. Great diagnostic accuracy of this point-of-care test for *Aspergillus* detection in ICU patients was illustrated in different studies, with sensitivity and specificity comparable to published data for ICU BALF GM testing. In particular, the significant high NPV of 96% may prevent overtreatment, and consequential treatment-related complications and development of drug resistance (178). Similar high NPV levels of BALF LFD tests were also reported in other studies in different patient cohorts (125, 157, 181).

Among the four participating hospitals in our analysis, sensitivities (between 75% to 100%) and specificities (between 78% to 95%) were relatively similar, with exception of Vienna, where a significant lower specificity was seen (54%). This statistical outlier could be explained by the differing inclusion criteria: in Vienna just samples that showed growing moulds were included retrospectively. The majority of those samples were obtained from patients without evidence of invasive aspergillosis. In contrast, most of the samples from Innsbruck were biobank specimens mainly from patients classified as probable IA. This variance, in combination with the small sample size of probable/proven IA cases, may clarify the differing sensitivities, positive predictive and negative predictive values among the four participating centres. In comparison, Graz and Mannheim

prospectively included all ICU patients who underwent BALF recovery for mycological workup. Hence, calculated PPVs were lower and NPVs were higher. These results may therefore reflect more a real-life intensive care setting than those from Vienna and Innsbruck. Nonetheless, we decided to additionally include the biobank samples from Innsbruck and the mould positive culture samples from Vienna for a higher number of probable and proven IA cases, which is always needed for a better interpretation of the diagnostic potential of a new test for a rare disease (178).

The BALF LFD test yielded a false positive in 20 patients with no evidence for IA in accordance to the EORTC/MSG criteria, which were originally developed for patients with haematological malignancies. This may be a reason for the underestimation of IA in ICU patients without haematological malignancies and classical risk factors. A part of these false positive LFD cases possibly resulted due to wrong classification and to BALF sampling conducted in an early stage of disease (178).

We slightly modified the 2008 revised MSG/EORTC criteria and added an “ICU stay above 4 days” as host factor, because host criteria focus on heavily immunosuppressed patients, but have not been evaluated in other non-neutropenic patients at risk for invasive aspergillosis (178). Blot et al. illustrated that EORTC/MSG host criteria are absent in approximately one third of proven IA cases in critically ill patients (183). However, all included participants in our study who fulfilled probable IA criteria had an ICU stay of a minimum of 10 days. The decision to widen the EORTC/MSG criteria to include “ICU stay above 4 days” might be more appropriate for patients in an ICU setting and was based on prior published data (development of IA was between 2 and 4 days) (178, 183, 220). However, it might be considered arbitrary. Hence, we also calculated the diagnostic performance of the LFD test when classifying IA based on the predefined EORTC/MSG definition (without “ICU stay above 4 days” as a host criteria) (69) and additionally on the previously published clinical algorithm (183). This algorithm has one main limitation: positive BALF culture for probable IA, which is known to be less sensitive. Finally, we found comparable test accuracy with consistent sensitivities and specificities among these three different classification criteria for IA (178).

A prospective study highlighted that the optimal diagnostic strategy for diagnosis of invasive aspergillosis should based on patient characteristics like underlying disease and leucocyte count. Non-neutropenic patients with haematological malignancies tend to be more frequently prone to airway-invasive aspergillosis, whereas angioinvasive aspergillosis was more often seen in acute leukaemia patients with leukocyte counts less

than 100/mm^3 (192). Accordingly, serum biomarker testing like LFD and GM can potentially lead to false negative test results in non-neutropenic ICU patients, and bronchoscopy with BALF sampling should be performed in case of suspected IA in these critically ill patients (178). The same was reported for the GM immunoassay: while the sensitivity of BALF GM was 88% in the ICU patient population, the sensitivity of the serum GM was with 42%, which was considerably lower (221). As mentioned above, bronchoscopy and BALF recovery is essential in non-neutropenic patients with IA suspicion. GM antigen detection is often not promptly available which could lead to a fatal delay of antifungal treatment initiation. LFD testing of BALF samples seems to be a credible alternative diagnostic tool to overcome this limitation in ICU patients at risk for IA. In our study, eight of the nine true positive BALF GM results yielded also a positive LFD test result, which was available within 15 minutes and without the need of a specialized equipped laboratory and/or highly trained staff. The sensitivity rate of conventional BALF culture in the secondary analysis was low, though significantly higher than in the main prospective study (50% versus 19%) (175, 178).

One limitation in our analysis is that the current gold standard for IA diagnosis, GM measurement in BALF, was not conducted in every included case. At this time the BALF GM index value seems to be most useful diagnostic tool for IA in critically ill ICU patients (221). Diagnosis of invasive aspergillosis based on positive BALF culture results as mycological criterion alone may induce misclassification of IA in some cases. This can be an explanation for the small variance of LFD sensitivities among the four participating hospitals. In our analysis, sensitivity of the LFD was higher in cases with a positive GM antigen detection than in those with positive culture result only and missing GM evaluation (178).

Another limitation of the study is that part of the BALF samples were initially frozen and stored at -70ºC. Indeed, former studies have shown great diagnostic performance of the BALF LFD when using previously frozen samples (125, 157, 181). The differences in patient characteristics such as underlying diseases, as well as the percentage of proven/probable IA among the participating centres, may be the most important limitation in the assessment of the LFD performance (178).

The impact of pre-emptive or targeted antifungal treatment on GM detection is well known. Negative GM results after AF treatment start may be a promising tool for estimation therapy response (222). In an animal model of Wiederhold et al. the BALF LFD test was less affected by antifungal treatment in comparison to serum GM and serum LFD (97). At

this time no other data on the impact of antifungal agents on the performance of the BALF LFD test exists. Therefore we performed the first analysis that evaluated the influence of mould-active agents on LFD performance in human BALF samples for the diagnosis of IA. Altogether 60 patients who were classified as probable or proven IA were included in the final analysis. The sensitivity of the LFD test was markedly lower in BALF samples of patients who were receiving systemic antifungal treatment at the time of the BALF sampling than in those who were not (52% versus 86%). Our data demonstrated that the BALF LFD performance may be very promising in untreated patients. In patients with ongoing mould-active prophylaxis/therapy negative BALF LFD as well as BALF GM results should be interpreted with caution (177). However, this point-of-care test may still be useful in patients receiving AF agents due to the high specificity of the test (99, 125, 157, 181).

In the same cohort, the BALF GM index and BALF culture were also reduced in samples that were obtained after antifungal treatment initiation. The sensitivities for BALF GM antigen detection when we used the cut-off 0.5 ODI were 71% in the presence of AF agents versus 95% in the absence of antifungal treatment; when we used the cut-off 1.0 ODI, 52% versus 81% (177). Decreasing sensitivity was also reported in a previously published multicentre prospective study. The sensitivity of BALF GM was significantly lower in patients who were treated with two or more systemic antifungals prior to bronchoscopy and BALF sampling compared to treatment-naive patients (194). Martens et al. observed a markedly decreased serum GM in patients with ongoing antifungal treatment and also a trend towards reduced BALF GM sensitivity (196). Marr et al. reported that the sensitivity may be more affected under targeted mould-active therapy than in patients receiving antifungal prophylaxis and empirical therapy (135). Similar results were seen in other studies evaluating the influence of antifungal prophylaxis (134, 189, 223).

The sensitivity of BALF culture was again significantly low in the overall study cohort (25%) and even lower in patients under systemic AF compared to those without (14% versus 31%). Our data indicate that the sensitivity of the BALF LFD test and BALF GM index may be decreased in the presence of mould-active AF treatment (177). Reduced sensitivity of BALF PCR has also been published by Reinwald et al.; sensitivity in this prospective multicentre study was 17% in presence of minimum two antifungal agents (163). Hence early and timely bronchoscopy is a crucial factor that influences the biomarker performance. Negative diagnostic test results can rule out suspected IA if the BALF sample was obtained in an early stage of the disease in the absence of mould-

active treatment. Whereas negative results in BALF samples, obtained during the presence of systemic antifungal medication, may indicate therapy response and may be a promising tool for treatment management (177).

A limitation in this retrospective analysis was the small number of patients receiving mould-active AF treatment, and the small percentage of false-negative LFD test results to conclude about the duration of AF treatment and false negative LFD results (177).

11 Conclusion

To sum it up, *Aspergillus*-specific and panfungal PCR may enable better species identification. Serial PCR testing in blood is recommend in cases of prior antifungal treatment. BALF recovery as sample from the direct site of infection is indispensable for diagnosis, if clinically feasible. Nonetheless no single diagnostic test provides a definite diagnosis of an invasive fungal infection. Our data illustrated that all the disadvantages and limitations of each diagnostic test as a single test can be compensated by test combination. The combination of microbiological culture in BALF, GM in BALF and serum with PCR resulted in a significant better diagnostic test performance and could improve diagnosis and management of invasive mould infection. In addition, same-day PCR and GM illustrated diagnostic efforts in patients at risk for invasive aspergillosis under mould-active therapy (175, 177, 178).

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