Dissertation

Novel and established biomarkers in invasive fungal infections in patients with hematological malignancies

submitted by

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Ich widme diese Arbeit den Patienten der hämatologischen Station an der Medizinischen Uni Graz, die ich in der Projektphase kennen lernen durfte

La gravitation de l'esprit nous fait tomber vers le haut…

Simone Weil

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 of this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Date Signature

Disclosure

Parts of the results of this thesis were published as full paper in August 2014 and in June 2016 the Journals Medical Mycoses (1) and Mycoses (2). Data from these publications were partly used within the thesis with permission of both Journals.

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Abbreviation

AIDS	Acquired Immune Deficiency Syndrome
ALL	Acute Lymphoblastic Leukemia
anti-TNFα	anti-tumor necrosis factor alpha
AML	Acute Myeloid Leukemia
ASCT	Allogenic Stem Cell Transplantation
BAL	Bronchoalveolar Lavage
BDG	1,3-β-D-Glucan
BMS	Bone Marrow Suppression
CGD	Chronic granulomatous disease
CLRs	C-type Lectin Receptor Family
CLSI	Clinical Laboratory Standards Institute
CMV	Cytomegalovirus
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computed Tomography
CVC	Central Venous Catheter
DC	Dendritic Cell
DGHO	Die Deutsche Gesellschaft für Hämatologie und Onkologie
DOR	Diagnostic Odds Ratio
EBA-2	Erythrocyte Binding Antibody 2
ECIL	European Conference of Information Literacy
ECV	Epidemiological Cutoff Value
EORTC	European Organization of Research and Treatment of Cancer
ESBL	Extended Spectrum of Beta-Lactamase Escherichia coli
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FN	False-negative
FP	False-positive
G-CSF	Granulocyte-Colony Stimulating Factor
GI tract	Gastrointestinal Tract
GM	Galactomannan
GVHD	Graft versus Host Disease
HAART	Highly Active Anti-Retroviral Therapy
HIV	Human Immunodeficiency Virus
HSCT	Hematopoietic Stem Cell Transplantation
IA	Invasive Aspergillosis
ICU	Intensive Care Unit
IFI	Invasive Fungal Infection
IFN	Interferon
IMI	Invasive Mold Infections
IPA	Invasive Pulmonary Aspergillosis
LFD	Lateral Flow Device
LAB	Liposomal Amphotericin B
MAB	Mannan Antibody
MAG	Mannan Antigen
MAM	Mannan/Anti-Mannan

MDS	Myelodysplastic Syndrome
MRSA	Methicillin Resistant Staphylococcus aureus
MSG	Mycology Study Group
MTA	Medical Technician Assistant
NADPH-Oxidase	Nicotinamide-Adenine-Dinuclide-Phosphate Oxidase
NLR	Negative Likelihood Ratio
NPV	Negative Predictive Value
ODI	Optical Density Index
PAMP	Pathogen-Associated Molecular Pattern
PCP	Pneumocystis Pneumonia
PCR	Polymerase Chain Reaction
PLB	Phospholipase B
PLR	Positive Likelihood Ratio
PPV	Positive Predictive Value
PRR	Pattern Recognition Receptor
Saps	Secreted aspartic Proteases
SD	Standard Deviation
SIRS	Systemic Inflammatory Response Syndrome
spp.	species
T4+	CD4+ T lymphocytes
T8+	CD8+ T lymphocytes
TCR	T-Cell Receptor
TEE	Transesophageal Echocardiography
Th17	T Helper Cell 17
TLR	Toll-like Receptor
TN	True-Negative
TP	True-Positive
TTE	Transthoracic Echocardiography

Abstract

Background: Invasive fungal infections (IFI) caused by *Aspergillus* and *Candida* are difficult to diagnose. They are associated with high mortality rates especially in patients with hematological malignancies. Newly developed biomarkers or diagnostic bundles may help to establish early diagnosis, especially when used in varying specimen.

Methods: This prospective and observational trial was performed at the Division of Hematology at the Medical University of Graz from July 2012 to May 2013. Patients at risk for IFI were included. Serum and urine samples were collected twice per week. Bronchoalveolar lavage (BAL) fluids were collected if ordered by the doctor in charge. Galactomannan (GM) antigen assay, mannan antigen (MAG) test, mannan antibody (MAB) test and 1,3-β-D-glucan (BDG) test were performed in all serum samples. GM assay was also assessed in urine samples. BAL fluid samples were tested for BDG assay, GM assay, and the novel *Aspergillus* Lateral Flow Device (LFD) test. Medical imaging and mycological laboratory work-up were performed if required and only ordered by the doctors in charge. We collected medical information of patients by chart review and compared it to the test results. According to *Aspergillus*, the participants were classified regarding the modified EORTC/MSG criteria.

Results: In total 103 patients approved consent, who made up for 160 cases. Eleven cases were classified as probable invasive pulmonary infection (IPA) and 22 as possible IPA. Three participants developed a candidemia. Regarding the GM assay a cutoff of 0.5ODI was used, except for urine samples, were we used a cutoff of 0.1ODI. It showed in serum samples the sensitivity, specificity, PPV and NPV of 63.6%, 87.9%, 36.8% and 95.6%. In BAL fluid samples the sensitivity, specificity, PPV and NPV was 83.3%, 100%, 100% and 94.7%. In urine samples, it showed sensitivity, specificity, PPV and NPV of 50%, 61.5%, 13% and 91.4%. Regarding the BDG assay, a cutoff of 80pg/ml was used. In serum, it had sensitivity, specificity, PPV and NPV of 54.5%, 35.1%, 7.5% and 88.9% in the *Aspergillus* subgroup and 100%, 38.6%, 3.7% and 100% in the *Candida* subgroup. In BAL fluids, the BDG assay showed a sensitivity, specificity, PPV, and NPV of 54.5%, 85.7%, 75% and 70.6%. According to the combined evaluation of MAG assay (cutoff >125pg/ml) and MAB assay (cutoff >10AU/ml) sensitivity, specificity, PPV and NPV of 0%, 96.9%, 0% and 98.4% were found. Due to invalid test charges of the *Aspergillus* LFD test, results were not evaluable.

Conclusions: Clinical and radiological findings are still the basis of diagnosis, but a pre-emptive initiation of antifungal therapy seems feasible with help of biomarkers. GM assay in urine samples or MAG assay test results in serum samples seem to be useful to rule out IA or candidiasis. BDG assay in BAL fluid samples was useful to recognize colonization with *Candida* but was not feasible to diagnose IPA. The GM assay in serum and BAL fluid samples confirms its value and the BDG assay is sensitive to diagnose candidemia.

Zusammenfassung

Hintergrund: Invasive Pilzinfektionen (IPI) sind schwierig zu diagnostizieren. Besonders bei PatientInnen mit hämatologischen Grunderkrankungen sind invasive Pilzinfektionen mit hohen Mortalitätsraten assoziiert. Neue Biomarker und die Verwendung verschiedener diagnostischer Bündel sollen künftig die Zeit zur Diagnose verkürzen.

Methoden: Diese klinische prospektive Studie wurde von Juli 2012 bis Mai 2013 an der Abteilung für Hämatologie, Universitätsklinikum Graz, Österreich durchgeführt. PatientInnen mit einem Risiko für IPI wurden in die Studie eingeschlossen. Zweimal wöchentlich wurden Serum- und Urinproben gesammelt und auf den GM Assay, MAG- und MAK Assay und BDG Assay getestet. Der GM Assay wurde zusätzlich in Urinproben durchgeführt. In BAL Proben wurden die Assays auf GM, BDG und *Aspergillus* LFD geprüft. Falls notwendig kam es zur Durchführung einer Bildgebung und einer Probenaufarbeitung bezüglich opportunistischer Pilze. Invasive Prozeduren wurden nur von den behandelnden ÄrztenInnen angefordert. Der klinische Verlauf wurde notiert und als Vergleich für die Testergebnisse herangezogen. Zudem wurden die PatientInnen strikt nach den überarbeiteten EORTC/MSG Kriterien eingeteilt.

Ergebnisse: Insgesamt nahmen 103 PatientInnen an der Studie teil, die 160 Fälle ausmachten. Elf PatientInnen hatten wahrscheinlich eine invasive pulmonale Aspergillose (IPA) und 22 eine mögliche IPA. Bei drei PatientInnen konnte via Blutkultur eine Candidämie nachgewiesen werden. Der GM Assay zeigte in Serum Proben eine Sensitivität, Spezifität, PPV und NPV von 63.6%, 87.9%, 36.8% und 95.6% und hatte in BAL Proben eine Sensitivität, Spezifität, PPV und NPV von 83.3%, 100%, 100% und 94.7%. In Harnproben lag die Sensitivität, Spezifität, PPV und NPV der GM Assay bei 50%, 61.5%, 13% und 91.45%. Außer bei Urinproben wurde ein Grenzwert von 0.5ODI verwendet. Im Urin einer von 0.1ODI. Im Serum zeigte der BDG Assay eine Sensitivität, Spezifität, PPV und NPV von 54.5%, 35.1%, 7.5% und 88.9% in der *Aspergillus* Subgruppe sowie 100%, 38.6%, 3.7% und 100% in der *Candida* Subgruppe. In BAL Proben lieferte der BDG Assay folgende Werte: 54.5%, 85.7%, 75% und 70.6%. Es wurde ein Grenzwert von 80pg/ml verwendet. Bezüglich der kombinierten Auswertung vom MAG- (Grenzwert 125pg/ml) und dem MAB Assay (Grenzwert 10AU/ml) fanden wir eine Sensitivität, Spezifität, PPV und NPV von 0%, 96.9%, 0%, und 98.4%. Die *Aspergillus* LFD konnte aufgrund einer fehlerhaften Charge nicht ausgewertet werden.

Schlussfolgerungen: Klinische Symptome und die Bildgebung sind noch immer Grundlage für die Diagnose einer IPI. Dennoch eignen sich Biomarker für ein präemptives Therapieschema überaus gut. Der GM Assay in Urinproben und der MAG Assay im Serum scheinen eine IA bzw. eine Candidämie ausschließen zu können. Der BDG Assay hingegen kann zwar die Kolonisation von *Candida* in BAL Proben nachweisen, ist aber für die Diagnose einer IPA wenig geeignet. Der GM Assay in Serum und BAL Proben bestätigt seinen Status als Diagnostikum und der BDG Assay ist sehr sensitiv zur Diagnose einer Candidämie.

1. Introduction

Invasive fungal infections (IFIs) are rare but fatal diseases with an increasing number of infections in recent decades.

Predisposing factors are immunosuppression (inherited, disease derived or induced by therapy) and disturbances of the skin and mucocutaneous barriers. Reasons for increasing rates of IFIs are multiple. Advances in medical care and treatment have improved survival of diseases that were associated with devastating mortality rates previously but increased the patient population susceptible for developing invasive fungal infections. Newly developed immunosuppressive therapies (such as chemotherapy, T-cell-modulation/suppression, long-term and high-dose therapy with corticosteroids), broad-spectrum antibiotics and indwelling devices (central venous line, urinary catheter, and ventilatory assistance) are found to be the leading causes of predisposing status. Furthermore, underlying diseases such as hematological malignancies, human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) and diabetes mellitus are also predisposing factors for IFIs [1].

Patient predispositions -- also called host factors -- constitute one important pillar that helps to diagnose fungal infections. The knowledge of clinical manifestations and epidemiology of fungal diseases in addition to well-functioning mycological laboratory are the other essential pillars of an early diagnosis. In fact, improved laboratory and clinical diagnostic work-up has resulted in an increased proportion of IFI diagnosed pre-mortem [2], also contributing to increasing IFI rates.

While research has focused on identifying ways of diagnosing different fungal infections to enable early initiation of targeted antifungal therapy, diagnosis is still challenging, interdisciplinary, and requires expert knowledge in addition to different radiographic and laboratory tools. In the routine clinical setting, it is difficult for medical doctors to distinguish between bacterial, viral, and fungal infections. Furthermore, *e.g.* an underlying hematological disease can mimic signs and symptoms of infection. Biomarkers for diagnosing IFI have been introduced in recent years and play a major role in diagnosis, but they do not ultimately prove the presence of an IFI. They only corroborate or mitigate the suspicion of an IFI and should always be interpreted together with radiological as well as clinical findings. Nevertheless, biomarkers play a major role in diagnosing IFI due to the lack of a well-functioning gold standard.

2. Human pathogenic fungi

All human pathogenic fungi have in common the ability to grow at temperatures ≥37°C (98.6°F) [3]. Primary invasive mycoses may occur in any human. Secondary invasive mycoses are caused by opportunists and occur mostly in immunosuppressed patients [4].

On hemato-oncological wards, the following fungal species are responsible for the vast majority of IFIs: *Aspergillus* species (spp.), *Candida* spp., and *Pneumocystis jirovecii* [3], as well as emerging molds, such as *Mucorales*, *Fusarium* and *Scedosporium*. Although many other species can cause IFIs, they may be less significant. This has been proved by a retrospective trial from Denmark performed in 2005, which showed that rarer yeast species cause only 1.1% of invasive infections [5].

In this dissertation thesis, the focus will be placed on invasive infections caused by *Aspergillus* and *Candida* in adult patients with underlying hematological malignancies.

2.1 *Aspergillus* species

Aspergillus is ubiquitous and grows indoors as well as outdoors in warm, humid and well-aerated materials [6]: in the ground, rocks, walls, wood, food as well as on compost - every material made of complex carbon, nitrogen, amino acids, and/or short peptides. It uses enzymes to break down the tissue and to absorb nutrients. Environmental issues (pH, nutrient factors, O_2, mass of collagen or elastin) play an important role in determining which genes are activated or inhibited during the growth phase [7] and thus determines the morphology.

Aspergillus is phylogenetically classified as Ascomycota Pezizomycotina Eurotiomycetes Eurotiomycetidae Eurotiales [8, 9]. It prefers asexual reproduction, which means the production of fast-growing, mitotic conidia. Hyphae grow septate and brachiate, generating millions of conidia at the stem of the mycelium, [10] which are airborne [11].

Aspergillus is very thermostable; therefore, it is able to grow at temperature up to 52°C (125.6°F) [1]. In approximately 90% of all cases, the subspecies *Aspergillus fumigatus* is responsible for invasive aspergillosis in humans, followed by *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus nidulans* [12].

2.1.1 Response of immune system to Aspergillus species

The main entrance point for *Aspergillus* is the respiratory system by means of deeply inhaling the 3- to 5-µm ubiquitous conidia. Disturbances of the skin barrier such as wounds or burns are also potential entry points [13]. Moreover, there are theories about the inhalation of contaminated water aerosols leading to *Aspergillus* infection [14]. The host also mounts various defense mechanisms to clear the invading fungus. The physiologic clearance by the respiratory epithelium may eliminate conidia. Once invaded, the resting/swelling conidia or hyphae are recognized by macrophages (TLR2, Dectin-1), DCs (TLR2, Dectin-1, TLR4, DC sign), epithelial cells (structurally diverse receptors), and TLR9. However, the morphological state of *Aspergillus* induces different host responses due to a varying set of PAMPs.

When *Aspergillus* is recognized by the innate immune system, an effective and rapid effector phase is carried out followed by a postponed but strong adaptive effector phase [15]. Directly after recognition, the complement system (C3, factor H [16], FHR1, FFHL1 [17]) and chemokines [18] decoy Th1, Th2, Th17, T_{reg} [19], memory T cells, and/or more phagocytes. In a trial by Garlanda et al., the lack of the opsonin pentraxin 3 have led to IPA in knock-out mice due to a decreased recognition by DCs and alveolar macrophages [20].

Th17 secretes IL22, which is important to control fungal growth [21].

Nevertheless, the neutrophil granulocytes carry out the main defense against *Aspergillus* conidia and hyphae by phagocytosis and secretion of oxidative intermediary agents [22]. A regulator of resistance and tolerance of *Aspergillus* is the indoleamine 2,3-dioxygenase, which is an immunomodulatory enzyme induced by IFNɣ and found on the cell surface of DCs and macrophages among others [23]. It inhibits the neutrophil granulocytes from protecting the tissue against excessive inflammation [19]. Due to this fact, a theory about a predisposition for IPA in patients with allergic pulmonary reactions to *Aspergillus* has been stated. Furthermore, in the blood system, the monocyte CD14+/CD16 plays a significant role due to phagocytosis, cytokine secretion (TNFα [24], CCr2-, Ly6C), and T4+ priming [25].

Natural killer cells are recruited by MCP1/CCL [26] and IFNɣ (macrophages) [27], and demonstrate direct activity against *Aspergillus fumigatus* [28].

Antibodies against *Aspergillus* are usually not sufficient for the defense or for playing a major role in diagnosis [29].

The proinflammatory response by TNF, IFNɣ, and IL6 on the one hand and the anti-inflammatory effects by IL4 and IL10 on the other hand are found to control the antifungal infection adequately [19].

Aspergillus can produce toxins such as aflatoxin, ochratoxine A, fumagillin, and gliotoxin. Gliotoxin inhibits the NADPH oxidase activation, macrophage ingestion and suppresses the functional T-cell response [30]. Aflatoxin is a secondary metabolite of *Aspergillus* and non-*Aspergillus* species. In the endoplasmic reticulum of hepatocytes, it is converted into reactive epoxides that build up mutagenic and carcinogenic adducts with DNA strands [31].

2.1.2 Clinical picture

In this chapter, we give a detailed overview of the signs and symptoms of invasive aspergilloses.

2.1.2.1 Invasive pulmonary aspergillosis

Invasive aspergillosis (IA) is defined as infection of at least one inner organ, and the disseminated form is defined as infections of at least two organs including the blood system as the source of spreading [32]. Invasive pulmonary infection with *Aspergillus* (IPA) is the most common manifestation of invasive aspergillosis (>80%) [10] in immunocompromised patients [1]. It can also occur in immunocompetent patients [33]. There are particular host factors that predispose to an IA/IPA [34], which will be described in the next chapter. Approximately, 200.000 IPA cases occur each year with an associated mortality rate of 30%- to 90% [35]. The broad range depends on varying factors, amongst others such as neutropenia [32] and initiation of the correct antifungal therapy in early stages of disease.

If the immune system´s response is not effective, inhaled conidia can germinate. The hyphae grow alongside the alveoli, penetrate into the vascular endothelium [36], and spread in the blood system with subsequent dissemination, constituting an invasive aspergillosis. The infection causes severe tissue destruction with infarction zones and necrosis that can be seen in biopsies. The *Aspergillus* tends to build-up nodules, which are a mixture of thallus (fungus-colony made from mycelium), phagocytes, and host tissue [1]. General symptoms can be fever, cough (productive or not), chest pain, hemoptysis, and dyspnea [32]. Though some patients do not show any symptoms at all during early stages of infection [10].

2.1.2.2 Disseminated aspergillosis

The signs and symptoms depend on the (at least two inner) organs involved, thus varying broadly and are mostly comparable to viral or bacterial infections. Generally every organ can be infected [9], but most frequently affected are the lung (50%- to 80%), heart (28%), kidneys (23%), and central nervous system (20%) [10, 37].

Dissemination shows also high mortality rates ranging from 50%- to 95% [38, 39] and the broad range depends also on whether appropriate antifungal therapy is initiated immediately.

2.1.3 Host factors of invasive aspergillosis

Host factors describe predispositions of an individual that affect their susceptibility to a disease. They are intrinsic factors (such as age, sex, race, behaviors) that influence the individual exposure, susceptibility or response to an agent. Eventually, they are useful to facilitate the diagnosis of diseases and can be innate or acquired.

The European Organization of Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) has developed a revised classification for IPA [34] that is applicable for patients with underlying hematological malignancies. This classification has been developed for scientific evaluation purposes only and was used to classify the participants of this trial.

The host factors for IPA are:

1. Neutropenia (<0.5 x 10^9/L) for longer than 10 days
2. Recent allogeneic stem cell transplantation
3. Therapy with corticosteroids at least 0.3mg/kg/day assessed as prednisone equivalent dosage for longer than 3 weeks
4. Recent therapy with T-cell suppressants or nucleoside analogues within the past 90 days
5. Inherited immune deficiency, such as chronic granulomatous diseases

One important host factor is cortisone that is administered frequently in higher dosages to hematological patients. Pathophysiological, it can decrease the oxidative mediated attack by macrophages directly and thus influence the defense of the immune system. In addition, cortisone supports the growth rate of *Aspergillus* up to 30%- to 40% and the cell synthesis for more than 150% [40].

2.1.4 Risk Factors of invasive aspergillosis

Risk factors are features that are associated with - and not causative for - diseases or their complications over the disease course. It is the increased probability to acquire a distinct disease. In comparison to the *Aspergillus* host, factors are the risk factors not so clear defined. Host factors and risk factors are not the same and must be distinguished clearly.

Risk classification	Risk factors for invasive aspergillosis
High risk >15% Incidence	1. allogeneic stem cell transplantation, especially when older than 40 years, underlying disease, relapsed disease, corticosteroid use 2. AML, especially when older than 40 years, high-dose cytarabine therapy, poor health condition, prior infection with *Aspergillus* and *Aspergillus* colonization 3. Long-term neutropenia (<0.5 x 10^9/L for longer than 10 days) 4. Prior *Aspergillus* infection 5. Colonization with *Aspergillus* species 6. Simultaneous CMV infection
Intermediate risk 5-15% Incidence	1. Neutropenia (< 0.5G/l for shorter than 10 days) 2. Signs and symptoms of *Aspergillus* infection despite broad-spectrum antibiotics 3. Colonization 4. Signs and symptoms of *Aspergillus* infection 5. Halo sign in high solution chest CT scan
Low risk <1% Incidence	1. Solid tumors 2. Short-term neutropenia
Additional Features	1. Heavy exposure to *Aspergillus* conidia by air or food 2. Diabetes mellitus

Table 1: Risk classification for invasive aspergillosis AML=Acute Myeloid Leukemia, CMV=Cytomegalovirus, CT=Computer Tomography. Reproduced from Lass-Flörl et al. [32]

2.1.5 Diagnosis of invasive aspergillosis

The diagnosis of IFI is difficult and requires expert knowledge. The basis of diagnosis consists of host factors, clinical evidence (which is not specific and must include medical imaging), and mycological features (combined with bacterial and viral work-up to rule out differential diagnosis) [34].

2.1.5.1 Classification of the IPA patient

Every patient at risk for IPA who matches the above-described host factors and shows clinical signs and symptoms for IPA or newly developed imaging findings that are compatible with IPA should be evaluated for *Aspergillus* infection. The patient´s classes are defined by revised EORTC/MSG criteria in 2008 and are presented in Table 2.

Classification	Definition
Possible IPA	1. Clinical/Radiological evidence (CT scan) compatible with IPA 2. Appropriate host factors are present 3. No mycological evidence present
Probable IPA	1. Positive test results of non-culture-based mycological laboratory tools, such as GM assay, BDG assay in serum, or positive culture from a non-sterile site (*e.g.* BAL) 2. Clinical/Radiological evidence (CT scan) compatible with IPA 3. Appropriate host factors
Proven IPA	1. Evidence of IPA, such as blood culture, histology including microscopy (*e.g.* stained smears, aspirate), and positive culture from a primary sterile site 2. Host factors, suspicious CT scan, and clinical evidence are not necessary

Table 2: Scientific classification of invasive pulmonary aspergillosis regarding the modified EORTC/MSG guidelines IPA=Invasive Pulmonary Aspergillosis, CT=Computed Tomography, BAL=Bronchoalveolar Lavage, GM=Galactomannan. Adapted from texts of Lass-Flörl et al. and De Pauw et al. and Barnes et al. [32, 34, 41]

2.1.5.2 Medical imaging

A CT scan can give valuable information about the involvement of the lung, although typical signs of IPA lack sensitivity and specificity [42], and do not frequently develop until the recovery of neutrophil granulocytes takes place [1]. For the diagnosis of IPA, a chest X-ray is only useful to a very limited extent due to its low resolution. Moreover, a high-contrast CT scan is necessary, because even conventional CT scans can falsely indicate a halo sign [43].

Typical signs for IPA include the “halo sign” [44] (Fig. 1) and the “air-crescent sign” (Fig. 2) [45]. These signs can also be caused by other microorganisms such as *Nocardia* spp. [1].

Nevertheless, a sensitivity of 87% and specificity of 57% for the halo sign have been reported [32]. Patients with neutropenic fever plus a halo sign have positive predictive value (PPV) for IPA of 80%- to 100% [12]. Other important radiological signs for IPA are pneumonic infarction, nodes close to the pleura, and cavernous changes [10].

If IPA is suspected, disease progression may be seen over the first seven days in CT scan, independently of neutrophil recovery, despite initiation of the correct antifungal therapy, and therefore, disease progression should be interpreted with caution in the early treatment period. Importantly, follow-up with CT scans should be performed and interpreted in the context of the patient's clinical development [43] but currently there is no established scheme when and how often the follow-ups should be performed.

2.1.5.3 Laboratory work-up: direct techniques

To diagnose an *Aspergillus* infection, culture and histopathology are important but collections of smears from deep tissues or biopsies from sterile sites are often hindered by the clinical conditions of patients [32] (such as thrombocytopenia or cardiorespiratory problems). Currently, bronchoscopy and bronchoalveolar lavage (BAL) are the main diagnostic methods but they require relative healthy patients and produce non-sterile samples. Another feasible, but not established, approach is the CT scan-guided percutaneous lung biopsy which is a relatively minimal invasive technique. In a study of 61 immunocompromised patients, biopsies were captured from newly developed nodules, opacities, halo signs, crescent signs and/or cavities as well as a bronchoscopy with BAL. Afterward a profound laboratory work-up was initiated. The CT scan itself showed a sensitivity of 100% and a specificity of 50%. Although sample contamination cannot be excluded perfectly, the sensitivity of *Aspergillus* PCR was 100% and the specificity 86% and the GM assay showed a sensitivity of 88% and a specificity of 94% respectively [46].

Once the sample has been obtained, laboratory work-up must be initiated. If material from the site of infection is collected, microscopic techniques may allow differentiation between fungal groups. The morphology is essential to distinguish *Aspergillus* from *Mucorales*, but cannot be used to differentiate between *Aspergillus*, *Fusarium*, and *Scedosporium* [47]. Fungi-Fluor™, Blancophor™ [48], or lactophenol cotton blue [49] are necessary to illuminate the conidia and hyphae for the diagnosis via histology.

Mycological culture enables identification at the species-level and allows also drug-susceptibility testing. *Aspergillus* hyphae grow rapidly within 24- to 72 hours on culture media. Pathogenic *Aspergillus* spp. can be distinguished from non-pathogenic species by

their ability to grow at temperatures higher than 37°C (98.6°F) [10]. If culture is frustrating, the infection will be classified as an invasive mold infection.

Unfortunately, conventional blood cultures have low sensitivity, sometimes displaying results after a long period of time [46] and remaining negative in nearly all cases of invasive aspergillosis [10, 32]. Nevertheless, a blood culture must be drawn to rule out bacterial co-infections. If the blood culture becomes positive for molds, *Aspergillus terreus* is most frequently detected [32]. In other sample materials, the culture shows a better performance than the blood culture, but sensitivity is rather low. For instance, in a study by Hoenigl *et al.* the mycological culture from BAL fluids showed a sensitivity and specificity of 50% and 95%, with a PPV of 71% and a negative predictive value (NPV) of 89% [50].

2.1.5.4 Laboratory work-up: indirect techniques

Non-culture-based biomarkers, such as GM assay, *Aspergillus* Lateral Flow Device (LFD) prototype, and 1,3-β-D-Glucan (BDG) assay are tools for the diagnosis of invasive aspergillosis [51] in serum and BAL fluid samples and will be discussed later in detail.

Polymerase Chain Reaction (PCR) is genus- or species-specific, depending on the chosen kit, but has not yet been standardized. Current PCR assays are relatively time consuming and expensive. It is described as a sensitive tool for diagnosis [52, 53] although this technique cannot distinguish infection from colonization [54]. For detailed information of the method, I refer to the instruction of use of the available kits.

2.2 *Candida* species

Out of 150 known species, only ten cause infections in humans: *Candida albicans*, *Candida guilliermondii*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*, *Candida kefyr*, *Candida lusitaniae*, *Candida dubliniensis*, *Candida glabrata* [10], and *Candida auris* [55].

Candida is a commensal of the human skin, mucosal surface, gastrointestinal tract (GI tract), genitourinary tract, and lung. Intestinal colonization is present in approximately 50% of healthy humans [56]. *Candida* species are polymorphic saprophytes, which favor the yeast form at room temperature. Under physiological conditions of their human hosts, they grow as hyphae or pseudo hyphae [1, 57].

Candida is a C-heterotroph and nutrients are incorporated by endocytosis. Typical metabolic pathways, such as glycolysis, the pentose phosphate pathway, the citric acid cycle, and the amino acid biosynthesis are present [58].

In general, they reproduce by budding, whereby mother cells can bud 20- to 30 times before they die. Daughter-cells are able to bud immediately [11]. Chlamydospores (asexual mitotic resting conidia) are thin-walled and range from 4- to 6 µm in diameter [10]. Therefore, they can lie down in smaller body spaces than the other forms. Chlamydospores are a characteristic of *Candida albicans* and *Candida dubliniensis* [59]. *Candida* is classified as a member of the Subkingdom Dikarya Ascomycota Saccharomycotina Saccharomycetes Saccharomycetales [8].

2.2.1 Response of immune system to Candida species

The undamaged skin and mucosa are important protectors against *Candida*, which is breached constantly by indwelling devices. Furthermore, broad-spectrum antibiotics can obliterate the physiological flora of the skin or intestine and lead to an overgrowth of such commensals. In addition, studies demonstrated that inhibitor factors (short-chain fatty acids, secondary bile acids) in the GI tract lead to a loss of the microflora and an aggravation of *Candida* colonization [60].

On the one hand, PRRs such as Dectin-1 (inducing the caspase-associated recruitment domain (CARD9) pathway) and Dectin-2 play an essential role for *Candida* recognition [61].

On the other hand, the virulence factors of *Candida* are as follows: (I) the polymorphism (especially the switch from yeast to hyphae) [58, 62, 63], (II) adhesins (ALS3 [64], Hwp1 [65]) and invasins (ALS3 [66], SSA1 [67]), (III) biofilm formation [68], (IV) contact sensing [69] and thigmotropism [70], (V) secreted aspartic hydrolases (Saps 1–10 [71], Phospholipase 5 (PLB5) [72], LIP8 [73]), (VI) pH [58], (VII) metabolic adaption [74], (VIII) environmental stress response [57], (IX) heat shock proteins [75] and small heat shock proteins [76], and (X) metal acquisition [77].

GI tract-derived invasive candidiasis occurs most common in patients with hematological malignancies, which among others lead to mortification of the GI epithelium due to aggressive chemotherapies.

After successful invasion, professional phagocytes constitute the first-line defense against the microorganism [64]. Neutrophil granulocytes are most potent in killing *Candida* independent of its morphology and primary entrance [78].

Despite this, *Candida albicans* can induce pyrophoric programmed cell death in macrophages in the first 6–8 hours as well as the transformation into a more robust hyphal form, within the first 24 hours after invasion. This helps to avoid phagocytosis and leads to macrophage death [79].

Th17 cells and its signature cytokines IL17 [80] and IL22 [61] are essential for anti-albicans defense and mucosal immunity [81].

Moreover, the humoral system supports the defense with complement factors and specific immunoglobulins. TNFα, IL6, and G-CSF are important to recruit neutrophil granulocytes [82]. Interestingly enough, myeloperoxidase deficiencies lead to an increasing count of infections with *Candida* [1].

2.2.2 Clinical pictures

In this chapter, we give a detailed overview of the signs and symptoms of invasive candidiases.

2.2.2.1 Candidemia

Candidemia is defined as the presence of *Candida* spp. in the blood system. Signs and symptoms range from fever only to sepsis [83]. Current studies show that the mortality rate ranges from 40%- to 60% [84] depending on the early initiation of correct therapy and age of the patient. It seems that the gut is the primary source of candidemia [85]. Other sources may be the environment, indwelling devices (Central Venous Catheter [CVC], Foley catheter, port-a-Cath systems) or medical products (blood concentrates). Noteworthy for clinical assessment, oral *Candida* colonization does not predict intestinal colonization [86]. If candidemia is detected, a thorough examination at the time of candidemia (including transthoracic echocardiography/transesophageal echocardiography [TTE/TEE], abdominal sonography, fundus control) is recommended [1] for diagnosis of disseminated infection [9].

2.2.2.2 Invasive candidiasis

Invasive candidiasis is defined as a *Candida* infection of at least one inner organ. *Candida* can infect every organ after candidemia or directly due to trauma, puncture or indwelling devices. The infected organ can eventually function as a reservoir with intermittent dissemination into the blood system. The signs and symptoms depend on the infected organ. *Candida* produces micro- and macro-abscesses [9] with an affinity for the eye, brain, myocardium, kidneys, and in neutropenic patients for the liver and spleen (i.e. hepatolienal candidiasis) [1].

In contrast to *Aspergillus* infections, which are in the lung (IPA) in more than 80% of cases, candidiasis of the respiratory tract is rarely seen. Respiratory tract candidiasis can occur as local or diffuse bronchopneumonia with multiple abscesses. Symptoms may be fever, productive cough, and shortness of breath [1]. For diagnosis, chest scanning and histopathology are important [32]. Generally, uncomplicated *Candida* colonization of the respiratory tract is common [1] even in neutropenic patients and must be kept in mind when interpreting diagnostic test results.

2.2.3 Risk factors of invasive candidiasis

For the definition of risk factors, I refer to page 7 of this thesis.

The *Candida* risk factors (not host factors) are as follows [32]:

1. Hospitalization in intensive care units (ICU)
2. Chemotherapy
3. Therapy with corticosteroids
4. Indwelling devices (such as intravascular catheters, central venous line, urinary catheter)
5. Total parenteral nutrition
6. Recent surgery, especially of the abdomen (involving the GI tract)
7. Long-term use of broad-spectrum antibiotics
8. Malignancies, HIV/AIDS, Diabetes mellitus
9. Neutropenia
10. Fungal colonization
11. Application of non-radiated blood products, association with the frequent administration
12. Renal insufficiency

2.2.4 Diagnosis of invasive candidiasis

Superficial *Candida* infections can be detected easily in scrapes and smears. Invasive forms require an elaborated work-up, including blood cultures, biomarker testing, medical imaging, and histopathology. In general, patients must be divided into different risk groups due to varying invasive candidiasis probabilities. Major patient groups at risk have/are (I) HIV/AIDS, (II) ICU dormant, (III) transplant recipients, (IV) patients with hematological malignancies, and (V) neonates [87]. For assessing the risk of invasive candidiasis, scores were invented by Leon *et al.* [88] and Ostrosky-Zeichner *et al.* [89]. These scores include for example *Candida* colonization and severe sepsis (Leon *et al.* only), surgery and total parental nutrition, use of broad spectrum antibiotics, immune suppressive agents, central venous catheter, any dialysis, the use of steroids, pancreatitis, an ICU stay for more than 72 hours (Ostrosky-Zeichner *et al.* only). Unfortunately, these scores were created primarily for non-neutropenic ICU patients and are therefore not applicable to patients with hematological malignancies.

2.2.4.1 Medical imaging

In scanning's and sonography of the liver or spleen, the "bull´s eye lesion" has a low sensitivity but still indicates an invasive candidiasis. Pathophysiological, these are macro- or micro-abscesses. They occur only until neutrophil recovery [1]. Other radiological signs, such as effusions, are not sensitive either and can also be caused by other microorganisms [1]. Another important medical imaging technique is the TTE/TEE to diagnose involvement of the heart.

2.2.4.2 Laboratory work-up: direct techniques

Blood cultures are the gold standard, but their sensitivity may not exceed 40%- to 60% [1]. Blood culture taken from a CVC or samples from non-sterile site (such as BAL fluids or sputum) may be false-positive due to colonization [1]. However, one single positive blood culture must be interpreted as proven candidemia and treated immediately [90]. If *Candida* infection is suspected, but blood cultures remain negative, biopsies, which are the basis of diagnosis, must be obtained. *Candida* culture from urine samples are rarely useful for diagnosing an IFI [91] due to the high rate of contamination and harmless colonization (30%) [1]. Yeasts can be seen easily in Gram stains, Fungi-Fluor™, and Blancophor™ [48].

2.2.4.3 Laboratory work-up: indirect techniques

Non-culture-based biomarkers which may be useful for the diagnosis of invasive candidiasis are the BDG assay, mannan antibody (MAB) assay, mannan antigen (MAG) assay [92] and will be discussed later in detail.

Candida PCR is not able to distinguish between infection and colonization [1] and has not been standardized yet. PCR should therefore be interpreted only in combination with other diagnostic tools and clinical evidence.

3. Epidemiology of invasive fungal infections in patients with hematological malignancies

In patients with hematological malignancies, IFIs are associated with high mortality rates. Early diagnosis is difficult to obtain and early initiation of correct antifungal therapy plays an important role in keeping the survival rate above 60%- to 80% [46, 93]. A risk classification for certain hemato-oncological diseases can help consider and interpret diagnostics (Table 3). The fact that mortality rates are high if treatment is not initiated in an early stage results in overtreatment with antifungal drugs (prophylaxis, pre-emptive or empirical therapy strategies). This can lead to the occurrence of unnecessary often rate of side effects and high costs.

Risk classification	Risk factors for invasive fungal infections
High risk	1. AML, especially during the induction or re-induction therapy 2. Patients during or after conditioning for allogeneic stem cell transplantation 3. Patients after heart-, lung-, and/or liver transplantation
Intermediate risk	1. Acute lymphatic leukemia 2. Chronic lymphatic leukemia 3. Chronic obstructive pulmonary disease 4. AIDS 5. Myelodysplastic syndrome
Low risk	1. Autologous stem cell transplantation 2. Hodgkin´s lymphoma 3. Chronic myeloproliferative diseases, especially chronic myeloid leukemia and Philadelphia-chromosome negative forms 4. Solid tumors 5. Myeloma 6. Single organ transplantation of kidney 7. Chronic immunologic diseases 8. Systemic lupus erythematosus

Table 3: Risk classification for invasive fungal infections AML=Acute Myeloid Leukemia, AIDS=Acquired Immune Deficiency Syndrome. Adapted from the text of Pagano, L., et al. [94]

3.1 Epidemiology of invasive mold infections

Invasive mold infections (IMI) are defined as infection caused by a not closer defined mold fungus. In an Austrian registry appraisal by Perkhofer *et al.*, IMI showed an incidence of 42 cases out of 1.000 patients per year [95]. In detail, these infections are mainly caused by *Aspergillus* (67%) followed by *Mucorales* (28%) [95], and *Fusarium* [96]. Hence, these fungal pathogens should optimally be included in the spectrum of antifungal prophylaxis.

The highest risk for IMI has been found in patients with acute myeloid lymphoma (AML) with 34% (annual risk of cases per 1.000 patients). Other patients at risk are those after

lung transplantation (17%), and a mixed residual patient group (14%) [95]. The main reasons for this are long-term immunosuppression due to the disease itself and its treatment. Patients after allogeneic stem cell transplantation (ASCT) have the highest risk for IMI in the first 100 days after transplantation, especially if T-cell recovery is slow, and if graft-versus-host-disease (GVHD) occurs [87].

Recent data from an epidemiological trial by Bitar *et al.* in 2014 showed that the incidence of invasive aspergillosis (increase of 2.7%) and mucormycosis [97] (increase of 8.7%) rose over a six year period in patients with hematological malignancies. In patients after hematopoietic stem cell transplantation (HSCT), the invasive aspergillosis also increased (plus of 9.8%) [98]. The explanation for this trend seems to be improved diagnostic approaches used by clinicians and microbiologists for the risk population. Reasons for a rise of mucormycoses, especially in HSCT patients, might be explained by the increased use of voriconazole prophylaxis (and also the use of itraconazole, micafungin, and caspofungin), which lacks efficacy against *Mucorales* [99], but also by the use of high-dose corticosteroids and anti-tumor necrosis factor alpha (anti-TNFα) therapy (such as infliximab) in patients with GVHD [100]. This must be considered in the rationale of therapy choice.

3.2 Epidemiology of invasive yeast infections

In patients with hemato-oncological malignancies, invasive yeast infection is mainly caused by *Candida*. In the absence of fungal prophylaxis, *Candida* is responsible for 70%- to 90% of all IFIs [32], which is the reason why every antifungal prophylactic approach must cover these microorganisms. Other causative yeasts might be *Trichosporon*, *Saccharomyces*, *Rhodotorula* or *Malassezia*; although these infections are very rare, thus the differentiation between *Candida albicans* and non-*albicans Candida* strains is more important.

In the before mentioned study by Bitar *et al.,* the incidence of invasive candidiasis increased over a six-year period in patients with hematological malignancies (plus of 4.3%) and HSCT (plus of 9.8%) [98]. The spectrum of *Candida* species varies in different patient groups. Interestingly, in hematological patients, a shift from *Candida albicans* (about 37.5%), toward other species, such as *Candida tropicalis* (about 17.5%), *Candida parapsilosis* (about 12.5%), *Candida glabrata* (about 10%), and others (around 21.25%) has been observed [101, 102]. This shift to non-albicans strains is explained by the use of antifungal drugs. If fluconazole is used, it will shift towards *Candida glabrata* and if caspofungin is used it will shift towards *Candida glabrata* and *Candida parapsilosis* [103].

Prophylaxis with posaconazole has also been reported to cause a shift toward non-albicans species [104], rare yeasts, and more resistant strains [103]. Nowadays, *Candida glabrata* is the cause about 24% of all fungal septicemias in the USA [105]. Thus, prior antifungal therapy must be included in the rationale of therapy choice in case of renewed suspicion for *Candida* infection. In the recent years, *Candida auris* shows increasing incidence with an increasing number of fatal cases in bacteremia's [106].

3.3 Antifungal therapy in patients with hematological malignancies

Countries, hospitals, and even wards [32] have different levels of fungal resistance. The routinely performed laboratory susceptibility tests monitor drug resistance and are important for initiating or adapting to correct treatment. They also depict the changing resistance patterns over time [107]. For susceptibility testing of fungal pathogens, appropriate clinical breakpoints are tested and recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical Laboratory Standards Institute (CLSI) [108]. For each fungal species-specific epidemiological cutoff, values (ECV) for different antimycotic drugs have been evaluated [109]. Breakpoints of antimycotic drugs approved by EUCAST and CLSI regarding susceptibility testing of *Candida* are documented annually by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) [110].

Currently, on hematological wards, the following scheme is the accepted procedure: First, the patient at high risk for IFI receives antifungal prophylaxis (posaconazole, voriconazole or fluconazole) and the physicians' monitor antifungal biomarker assays (such as the GM assay) twice per week. Second, if the patient´s health condition worsens (*e.g.* fever, fever again, tachypnea, signs and symptoms of sepsis), the entire diagnostic cascade must be started to enable early initiation of the correct therapy. Diagnostic procedures include medical imaging such as the X-ray of the chest, CT scan, fungal biomarkers, blood cultures, bronchoscopy to obtain BAL fluid specimen, and further microbiological work-up. When microbiological and histopathological work-up shows evidence of IFI, targeted or preemptive therapy will be initiated.

Pattern	Definition
Prophylaxis	Primary (avoid an IFI) or secondary (defined as condition after fungal infection) treatment to avoid infection in patients who are at high risk for IFI (Table 2) and in hazardous phases, such as neutropenia or low T-cell count. Regarding the EORTC/MSG criteria, no evidence of IFI is present at the time of prophylaxis initiation (no clinical evidence, no suspicious CT scan, no positive fungal biomarker)
Empirical therapy	Treatment due to clinical suspicion, such as long-term fever despite broad-spectrum antibiotics for longer than 14 days, in patients who match the host factors. Regarding the EORTC/MSG criteria, no evidence of IFI is present (no positive fungal biomarkers nor suspicious CT scan are present)
Preemptive therapy	Treatments due to clinical evidence or indirect fungal signs and positive fungal biomarkers or suspicious CT scan are present. Patients have possible or probable IFI according to the EORTC/MSG criteria
Targeted therapy	Proven fungal infection (positive culture, histology) from a sterile site. Clinical evidence, fungal biomarkers, and CT scans are not affecting the start of the definitive therapy.

Table 4: Definitions of therapeutic patterns for fungal infections IFI=Invasive Fungal Infections, EORTC=European Organization for Research and Treatment of Cancer, MSG=Mycology Study Group, CT=Computed Tomography. Reproduced from the texts of De Pauw, B., et al. and Barnes, R.A. et al. [34, 41]

As soon as the patient's health condition has improved, a well-considered step-down of the antifungal therapy is recommended [92]. In some cases, secondary prophylaxis is feasible and should be considered. The differentiation between fungal, viral, and bacterial genesis as well as underlying disease mimicking inflammation is the challenge.

3.3.1 Antifungal prophylaxis

Many prospective clinical trials have been performed in order to find the best antimycotic drug for prophylaxis during high-risk phases for IFI. Today, the "European Conference of Information Literacy" (ECIL) 5 [111] and "Die Deutsche Gesellschaft für Hämatologie und Onkologie" (DGHO) [112] recommended posaconazole as first-line prophylaxis in AML patients during induction therapy. The posaconazole plasma level should be controlled at least once a week if the suspension is used. Recent trials showed that the pill results in higher posaconazole blood levels , seemingly due to a superior absorption [113].

Posaconazole oral suspension remains the drug of choice for IFI prevention in patient with chemotherapy induced neutropenia in AML, myelodysplastic syndrome (MDS), and allogeneic HSCT recipients with GVHD. We strongly recommend posaconazole prophylaxis in these patient populations as standard of care (AI) [112]. The recommendation of itraconazole prophylaxis remains unchanged. It is a poor strength

recommendation (CI) for transplant recipients and in neutropenia settings. Common side effects of itraconazole are stomach pain, nausea, dyspepsia, and a bad taste in the mouth [32].

3.3.2 Antifungal therapy

Proven IPA should be treated with isavuconazole (AI) or voriconazole (AI), or liposomal amphotericin b (LAB) (BII) in patients with expected or developed long-term neutropenia. In patients after allogeneic HSCT (and with neutropenia), or in patients after allogeneic HSCT or other patient groups without neutropenia a treatment with isavuconazole (AII), voriconazole (AII), or LAB ((BII) is recommended information on the treatment of extrapulmonary disease and the treatment of the fever-driven approach, we refer to ESCMID guideline from 2017 [Ullmann, 2018] The major exception is fluconazole, for which *Aspergillus* spp. are intrinsically resistant [32]. Single resistance mechanisms against azoles have been described [114, 115]. *Aspergillus fumigatus* azole resistance ranges, depending on the region, from 1.7%- to 6.0%. Reasons for the different regional resistance patterns may be the wide use of azoles in hospitals, as well as on animal farms, and in agriculture [115]. Isavuconazole has been invented relatively new and, due to the effectiveness comparable to voriconazole but with less side effects, changed the guidelines in therapy of patients with proven or highly suspected IPA [116].

The first-line therapy for candidemia in patients with malignancies is fluconazole and LAB [32]. Depending on the subspecies, found recommendations for treatment vary [110].

In cases of a breakthrough infection are defined as infections, which occur under antifungal infection. Reasons may be too low drug levels in blood or resistances. The fungal spectrum may change to rarer fungi, such as *Scedosporium prolificans* [117], *Trichosporon asahii* [118], and *Mucorales* [119], which are often not covered by antifungal prophylaxis. A selection towards *Scedosporium prolificans* occurs if posaconazole or voriconazole is used and towards *Mucorales* if voriconazole is used [32].

4. Hypotheses

In this study, fungal-specific biomarkers for the early diagnosis in IFIs were evaluated in patients with hematological malignancies. Biomarkers were chosen for their relevance and promise in previous publications:

1. Performance of GM assay test results in urine samples is comparable to that of the GM assay test results in serum and BAL fluid samples to diagnose invasive aspergillosis
2. The combination of MAB and MAG assay testing is useful for the early diagnosis of invasive candidiasis
3. Performance of the *Aspergillus* LFD prototype test results is comparable to that of the GM assay test results in BAL fluids and serum to diagnose IPAs
4. BDG assay levels in BAL fluid samples are useful for the early diagnosis of IFI and/or to rule out pulmonary IFI

The evaluated assays were:

1. *Aspergillus* Lateral Flow Device antigen test (targeting JF5)
2. Platelia™ *Aspergillus* Antigen Assay by Bio-Rad (targeting GM)
3. Fungitell 1,3-β-D-Glucan assay by CAPE COD Diagnostics
4. Platelia™ *Candida* Antibody Plus assay (IgG/IgM/IgA mannan) by Bio-Rad
5. Platelia™ *Candida* Antigen Plus assay (targeting mannan) by Bio-Rad

This study focused on the early diagnosis of IFI and the tests were performed in different sample types described as follows: Serum, BAL fluids, and urine samples.

And the following statistical target values were evaluated:

1. Sensitivity
2. Specificity
3. NPV
4. PPV

5. Material and methods

In this chapter, we present the material and methods of the “IFI” study.

5.1 Study population

This clinical prospective study was conducted between July 2012 and May 2013 at the University hospital of Graz, Austria. Patients hospitalized at the Division of Hematology and at the Department of Internal Medicine were observed during daily clinical rounds. Patients with underlying hematological malignancies who match the host factors for IFI (Page 8, Table 1), and/or show clinical signs of IFI were screened for study inclusion. A signed informed consent form, obtained from every participant after detailed explanations, forms the basis of sample and data collection. The idea and design of the study is developed by PD Dr. med. univ. Martin Hönigl from the Section of Infectious Diseases and Tropical Medicine. Additionally, the Institute of Hygiene, Microbiology, and Environmental Medicine as well as the Clinical Institute of Medical and Chemical Laboratory Diagnostics cooperated were part of this trial. The study adhered to the 1996 Declaration of Helsinki, Good Clinical Practice, and the local ethics committee of the Medical University of Graz (protocol number 23–343) approved the study protocol.

The inclusion criteria were developed by using the *Aspergillus* host factors of the revised EORTC/MSG guidelines and the risk factors for an IFI.

Inclusion criteria:

1. *Aspergillus* host factors and risk factors for IFI and/or clinical/radiological signs suspicious for IFI
2. Age ≥18 years

Exclusion criteria:

1. No informed consent
2. Age ≤18 years

All participants who were asked to participate in the study received individual study ID numbers. Patients who declined to participate in the study also received an ID number. Participants who were admitted more than one time during the study received a new ID number each time. This is done for reasons of clarity and comprehensibility. All participants were screened for fungal infections caused by other genera, such as

Pneumocystis, *Mucor*, and *Cryptococcus* as well as for bacterial infection to draw pure *Aspergillus* and *Candida* based conclusions.

5.2 Sample collection

Two sera, one EDTA, one Lithium tube, and one urine sample were collected from every participant twice per week simultaneously and routinely. All samples were frozen in the study freezer of the Section of Infectious Diseases and Tropical Medicine (-70°C; -94°F), meticulously documented, and matched to the ID number of the participant as basis of the biobank.

The principal investigator accompanied all participants who received routine bronchoscopy with BAL. Every bronchoscopy/BAL was conducted due to newly emerged pulmonic infiltrates seen in chest scanning. Routine aerobe and anaerobe blood cultures were drawn in case of fever or other symptoms of infections. A chest CT scan was routinely ordered in cases of clinical signs suspicious of pulmonary infection in patients with long-term fever despite broad-spectrum antibiotics. No bronchoscopy, CT scan, or X-ray was ordered by the principal investigator and thus in the frame of this study.

Retrospective performed assays	Prospective performed assays
Mannan antibody in serum	GM assay in serum
Mannan antigen serum	BDG assay in serum
BDG assay in BAL fluid samples	GM assay in urine samples*
	Aspergillus LFD samples in BAL fluids
	GM assay in BAL fluid samples

Table 5: Biomarker tested retrospectively or prospectively during the IFI study *galactomannan assay in urine samples was tested prospectively for one kit (07/2012-08/2012), afterward it was tested retrospectively if the same day GM assay test result was higher than 0.3 ODI in serum. GM=Galactomannan, BDG=1,3-beta-D-glucan, BAL=Bronchoalveolar Lavage, LFD=Lateral Flow Device Test, IFI=Invasive Fungal Infection

Within the scope of this trial the GM assay, BDG assay, and MAG/MAB assays were conducted on each serum sample. Furthermore, the GM assay was performed on each urine sample within 07/2012-08/2012 (1°^{xx}°Kit). Afterwards, urine samples were tested only if the corresponding GM test results in the serum samples are ≥0.3 ODI (Optical Density Index). Moreover, the GM assay, BDG assay, and tests with the *Aspergillus* LFD prototype were performed in each BAL fluid sample. Beyond the scope of this trial, a standard microbiological work-up was performed. According to the BAL fluids, the *Pneumocystis jirovecii* antigen tests, and, if necessary, the *Cryptococcus* antigen test was conducted. Both tests were part of the clinical routine.

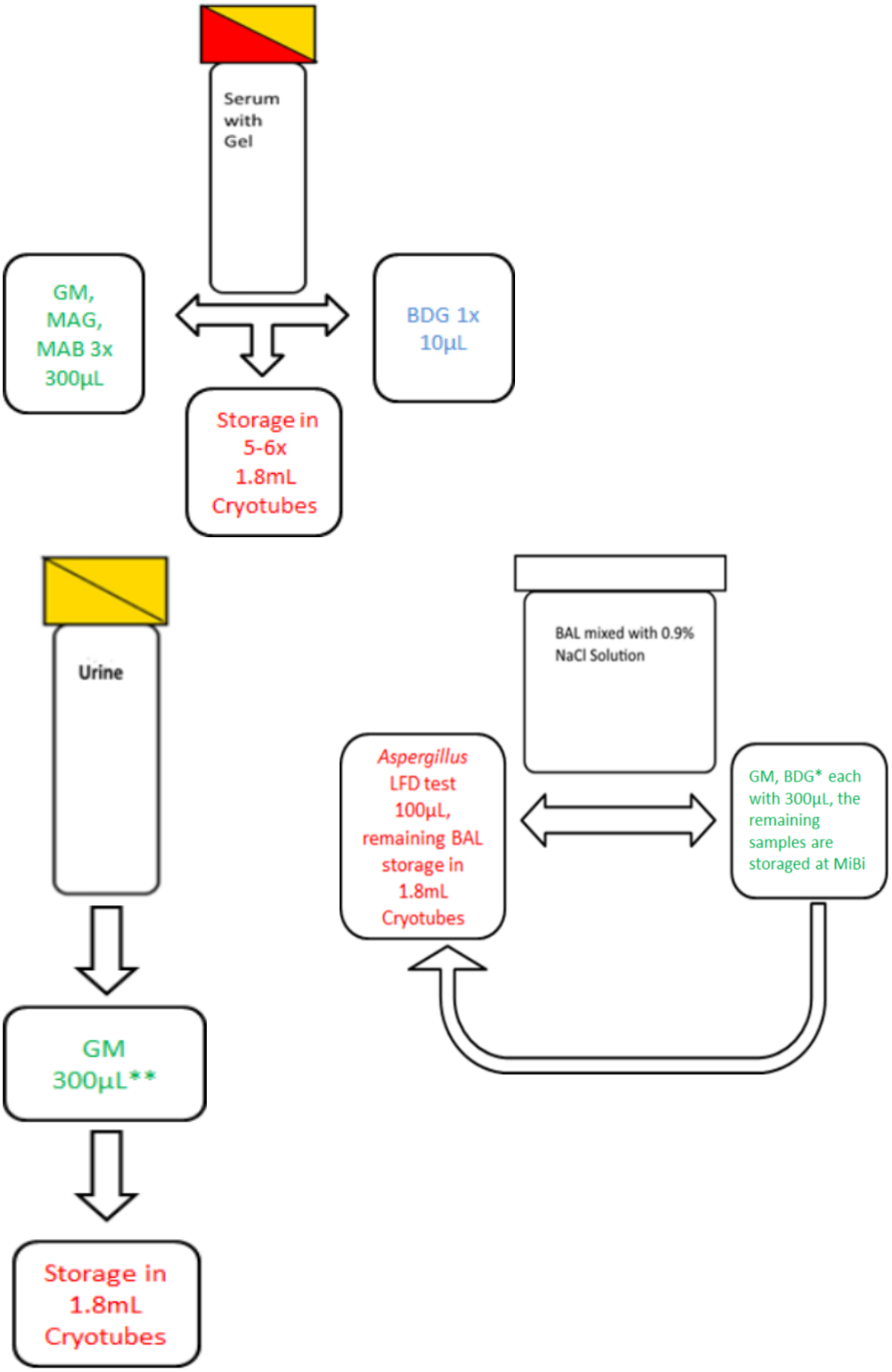

Figure 1: Processing pattern of the samples Green=Sample processing at Institute for Hygiene, Microbiology, and Environmental Medicine, Blue=Sample processing at Clinical Institute of Medical and Chemical Laboratory Diagnostics, Red=Laboratory of Section of Infectious Diseases and Tropical Medicine, *tests were performed retrospectively **GM assay was tested in each urine sample prospectively for one kit, afterward only samples with positive GM assay test result in serum were also tested for GM in urine; GM=Galactomannan

5.3 Laboratory testing

One important object was the laboratory testing. In the following chapter, we present the laboratory procedures of the biomarkers.

5.3.1 Galactomannan enzyme immunoassay

The GM assay is a qualitative immunoenzymatically sandwich microplate assay, called Platelia™ *Aspergillus* Ag, which is produced by Bio-Rad (Hercules, California, USA). The detection of GM is CE/IVD approved for serum and BAL fluid samples. For early diagnosis, Bio-Rad recommends performing the assay twice weekly on the serum of patients at risk for invasive aspergillosis [120].

Essentially, the assay uses the monoclonal rat erythrocyte binding antibody 2 (EBA-2), which binds on the 1,5-β-D-galactofuranoside of the immunodominant epitope on the GM antigen. The EBA-2 antibody is placed in each microplate well to bind GM and is used to build-up a complex with the GM and a peroxidase to sensitize the microplate. To avoid interactions and dissociation from serum proteins and immune complexes (*i.e.*, peroxidase-linked EBA-2 antibody), the sample must be freed by heat. After the heating process, the sample must be incubated for 90 minutes at 37°C (98.6°F) with the fixed monoclonal antibody. After incubation, the samples must be washed (to free them from unbound materials) with a special washing program for this kit. Afterwards, a chromogenic tetramethylbenzidine solution must be added to each well to create measurable staining. After a 30-minutes incubation period (in the dark), staining must be stopped by an acid (that changes the color from blue to yellow). Finally, the results can be measured with a spectrophotometer at wavelength 450nm and 620/630nm [120].

In this study, we performed serum sample collection twice weekly with sterile vacuum tubes that allow clotting. We stored the samples at 2°C- to 8°C and completed the GM assays no later than on day three after collection. We froze the remaining serums of the samples at -70°C and we avoided freeze-thaw cycles.

BAL fluid samples were collected in sterile saline solution. We stored the BAL fluids at 2°C- to 8°C but the GM assay was performed no later than on day three after collection. Eventually, we froze the remaining BAL fluid samples at -70°C (study freezer) and avoided freeze-thaw cycles.

We collected the urine samples in sterile tubes. We stored the samples at 2°C- to 8°C, but again, we performed the GM assay no later than on day three after collection for 96

samples (one kit) from 07/2012 to 08/2012. Afterward, we tested only urine samples from patients with a GM test result >0.3ODI in serum samples taken on the same day. Finally, we froze all urine samples at -70°C and we avoided freeze-thaw cycles.

Study results proposed varying ODI cutoffs in BAL fluid samples depending on the underlying conditions, such as hematological diseases or recipient of lung transplantation. In this study, we used the same cutoff as in serum samples, following recommendations from Bio-Rad. The optimal ODI cutoff in urine samples is not yet established. In a study by Duettmann *et al.* a cutoff of 0.1ODI was suggested with the best sensitivity of 47.6% (and specificity of 86%) [121]. We could confirm this cutoff in our samples set. We had a slightly different sensitivity and specificity of 50% and 61.5% and a PPV and NPV of 13% and 91.4%.

5.3.2 Aspergillus lateral flow device test

The *Aspergillus* LFD prototype is an immunochromatographic assay that uses the lateral flow device principle. It is developed by C.R. Thornton from University of Exeter/UK and is based on a mouse hybridoma cell line that secrets an *Aspergillus* murine monoclonal antibody called JF5 (IgG_3). The test requires minimal training to perform. The test is developed for patients at high risk of invasive aspergillosis in order to enable a rapid diagnosis and is evaluated for serum samples [122] and BAL fluid samples [123]. Urine or other specimens have not been evaluated yet.

An immune-fluorescence evaluation of JF5 antibody showed that it is held and secreted on the hyphal surface of *Aspergillus fumigatus*. Further investigations with immunogold electron microscopy exposed that the JF5 antibody is a part of the hyphal cell wall, the septa, and the capsule-like layer surrounding each cell. The antibody is not found on the surface of non-germinated conidia [122]. Regarding the test principle, the JF5 antibody is immobilized in a capture zone on a porous nitrocellulose membrane. Conjugated and colloidal gold particles with the JF5 IgG serve as a detection reagent. The antibody-gold conjugate is placed at the release port to bind the antigen. When the solution passes along the porous membrane by capillary action, it will bind to the immobilized antibody in the capture zone [124].

In this trial, we applied 100µL of the naive BAL fluids into the release port of the LFD test device. The test result is available within 10- to 15 minutes, but we did not read it after more than 15 minutes to avoid false-positive test results [123, 125]. We tested BAL fluid samples directly after bronchoscopy. The intensity of the test line compared to the control

line gives the result: weak (+), moderate (++), strong positive (+++) or negative (-) [123]. Regardless of insensitivity, a reddish changing test line displays the presence of the JF5 antigen [124].

5.3.3 1,3-β-D-glucan assay

The Fungitell BDG assay is a qualitative protease zymogen-based colorimetric assay used in patients at risk for IFI. Associates of CAPE COD Inc. (East Falmouth, MA, USA) produces it. The test result informs about deep-seated mycoses and fungemia but is considered as a pan-fungal marker and cannot differentiate between the causative fungal species. It is CE/IVD approved for use in serum samples [126].

The Fungitell kit is designed for the simultaneous testing of 21 samples. The original protocol lacks automated procedures and rapid single-sample testing. Prüller *et al.* invented in 2012 an automated protocol based on of the blood coagulation cascade, which is used in this trial to detect BDG in serum samples. The corresponding paper was published in 2014 [127].

The Fungitell test principles base on a modified limulus amebocyte lysate pathway. Modification refers to the exchange of factor C with factor G. BDG stimulates factor G (a serine protease zymogen) which converts pre-clotting enzymes into clotting enzymes. These in turn break down an artificial chromogenic peptide substrate (Boc-Leu-Gly-Arg-pNA) to Boc-Leu-Gly-Arg + pNA. The emerging chromophores absorb at a wavelength of 405 nm. The test result is based on the determination of the increased optical density rate in samples [126].

BDG is a major cell-wall component of various medically important fungi including produced by *Candida*, *Aspergillus*, *Fusarium*, *Trichosporon*, *Saccharomyces cerevisiae*, *Acremonium*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii*, and *Pneumocystis jirovecii*. Infections caused by *Cryptococcus* and *Mucorales* produce only low levels of BDG on the surface. *Blastomyces dermatitidis* does not have BDG on its cell-wall in its monocellular yeast form [126].

In this study, we performed the sample collection with sterile vacuettes from Greiner Bio-one that enable clotting. Serum samples were stored temporarily at 2°C- to 8°C, and determined within a maximum of three days, or otherwise frozen at -20°C (freezer of laboratory). We performed the BDG assay not later than six months after freezing. The remaining samples were frozen at -20°C. We avoided freeze-thaw cycles. In case of clotting processes in the tube that interfered with test results samples had to be retested.

The BDG assay was also performed on BAL fluids retrospectively not later than six month after freezing at -70°C. As mentioned above, we collected the samples in sterile tubes and in saline solution (0.9% NaCl).

Associates of CAPE COD Inc. recommend interpretation of test results using the following cutoffs: <60pg/ml as negative, from 60pg/ml- to 79pg/ml as possible infection (additional sampling and testing of sera is recommended), and ≥80pg/ml as positive. We used the last mentioned cutoff to interpret our test results as positive in the context of signs and symptoms as well as medical imaging [126].

5.3.4 Anti-mannan antibody assay

The mannan antibody (MAB) assay is an indirect immunoenzymatically microplate assay for the detection of mannan Ig antibodies and is approved in serum as well as plasma samples. The Platelia™ *Candida* Ab Plus, is produced by Bio-Rad (Hercules, California, USA). It is recommended that the test results be interpreted in conjugation with the Platelia™ *Candida* Ag Plus assay to improve early diagnosis and sensitivity [128].

The test principle and protocol are as follows: the samples are diluted and dispensed into test plate wells, which are coated with mannan from *Candida albicans*. After an incubation phase of 90 minutes at 37°C, peroxidase-conjugated goat anti-human IgG/IgA/IgM polyclonal antibodies are added, and the sample is incubated again for 60 minutes at 37°C. After a washing process, the samples are incubated with peroxidase substrate for 30 minutes, in the dark at room temperature, and the reaction is finally stopped by addition of 1N sulfuric acid. In the end, the absorbance is measured with a spectrophotometer set at a wavelength of 450/620nm [128].

In this study, we performed the sample collection with sterile serum tubes. We stored the samples temporarily at -70°C (study freezer) for six months and performed the tests within one day after opening the sterile tube. We avoided freeze-thaw cycles and protected the samples from heat.

Test results <5AU/mL were interpreted as negative, from 5Au/ml- to 10AU/mL as intermediate, and test results ≥10AU/mL as positive for mannan antibodies [128].

5.3.5 Mannan antigen enzyme immunoassay

The mannan antigen (MAG) assay is a one-stage immunoenzymatically sandwich microplate assay for the detection of mannan antigen in patients at risk for invasive candidiasis, approved for use in serum or plasma samples. The Platelia™ *Candida* Ag Plus is produced by Bio-Rad (Hercules, California, USA). For the correct diagnosis a combination of this test and the MAB assay is recommended for early diagnosis of *Candida* infection as well as monitoring of patients [129].

Essentially, the assay uses the monoclonal antibody EBCA1 to target *Candida* α, 1-5,oligomannosides. Each test plate well bottom is coated with the antibody so that it can bind the peroxidase labeled EBCA1. For dissociation and precipitation from immune complexes and serum proteins, the samples must be treated with heat. After an incubation period (90 minutes at 37°C) and washing program, the complexes need time to bind to the chromogen solution (30 minutes in the dark at room temperature), after stopping this binding process with 1N sulfuric acid, the assay can be read at a wavelength of 450/620nm [129].

In this study, the specimens were collected in sterile serum vacuum tubes. We stored samples at -70°C for six months, but we performed all assays for the trial within one day. For the biobank, we froze the samples at -70°C, and freeze-thaw cycles were avoided.

Test results <62.5pg/ml were interpreted as negative, ranging from 62.5pg/ml- to 125pg/ml as intermediate, and ≥125pg/ml as positive for MAG [129].

5.4 Software

We performed the statistical analysis with SPSS Statistics 21 by IBM. We collected data and created tables with help of Excel 2010 by Microsoft. We wrote the thesis in Word 2010 by Microsoft and designed graphs with Power Point 2010, Paint.NET, and Excel 2010 by Microsoft.

5.5 Statistical analysis

The statistical analyses include decisions on how to interpret data and on how to deal with it. In the following chapter, we present our strategies.

5.5.1 Descriptive analysis

We performed a profound description of the study population. Additionally, we put our focus on the different fungi and created an (1) *Aspergillus* and a (2) *Candida* subgroup. BDG assay test results had an interim position because it can detect both fungi and, thus, were included in both subgroup analyses.

(1) For the statistical analyses of the biomarkers regarding the aspect "*Aspergillus*" (GM assay, *Aspergillus* LFD prototype, but also BDG assay) the participants were divided in three subgroups:

1. includes all cases which matched the *Aspergillus* host factors excluding those with proven invasive candidiasis
2. includes all cases, which were classified per the modified EORTC/MSG criteria as possible cases and excluding those of proven invasive candidiasis (see page 8, Table 2)
3. includes all cases, which were classified per the modified EORTC/MSG criteria as probable cases and excluding those of proven invasive candidiasis (see page 8, Table 2)

The rationale for this subgroup analyses was to use a pattern driven approach (clinical signs and symptoms of infection) in a study population with broad-spectrum of underlying hematological diseases and treatments as it is common at hematological ward (observational aspect of study).

(2) For the statistical analyses of the "*Candida*" subgroup the MAG- and the MAB assays were evaluated only in the following subgroup, which includes participants, who matched the risk factors for *Candida* infection by deducting the cases which were classified as probable/proven IPA as per the modified EORTC/MSG classification. The BDG assay test results were considered, too.

1. Patients matching the risk factors of invasive *Candida* infection excluding those classified as probable IA/IPA per the modified EORT/MSG guidelines
2. Proven colonization with *Candida* strains (any site is considered) excluding those classified as probable IA/IPA per the modified EORT/MSG guidelines
3. Proven candidiasis (blood culture) excluding those classified as probable IA/IPA per the modified EORT/MSG guidelines Furthermore, we evaluated the biomarkers sample-based or case-based, and depending on the hypothesis and the number of samples, we had. In whole, we preferred the case-based approach because it gives a more realistic picture of the procedures performed in hospitals.

Participants who matched the *Aspergillus* host factors for IA/IPA were classified per the modified EORTC/MSG recommendations. We decided to make the following exceptions:

1. Participants who did not match the *Aspergillus* host factors but had an absolute suspicious chest CT scan and positive test results for GM assay/BDG assay in the BAL fluid sample. They were classified as probable cases
2. Participants with an acute onset of worsened health condition who were severely suspicious for an IFI, but only chest X-rays had been performed due to practical reasons (such as isolation because of neutropenic phase), were excluded from further evaluations.

If a BAL fluid samples was negative for GM assay but showed by microscopy or culture fungal growth, the sample was classified as negative regarding IPA (GM assay) and instead as positive regarding IFI (BDG assay).

	Test result from…	… are compared with:
1.	*Aspergillus* LFD prototype test in BAL fluid samples	GM assay test results in BAL fluids and serum samples
2.	GM assay test result in urine samples	GM assay test results in BAL fluids and serum samples

Table 6: Overview of the compared biomarker assay test results in the different specimens
LFD=Later Flow Device, BAL=Bronchoalveolar Lavage, GM=Galactomannan

6. Results

In this chapter, we highlight the descriptive evaluation of the study population.

6.1 Common characterization

During the study period, 212 cases matched the inclusion criteria and were screened for inclusion in the study. A total of 52 (24.5%) cases refused to give informed consent. Hence, a total of 160 cases were included in the study, representing 103 individual patients. Thirty-three patients participated more than once with an average of 2.72 participations. In total, these 33 patients comprised 90 cases.

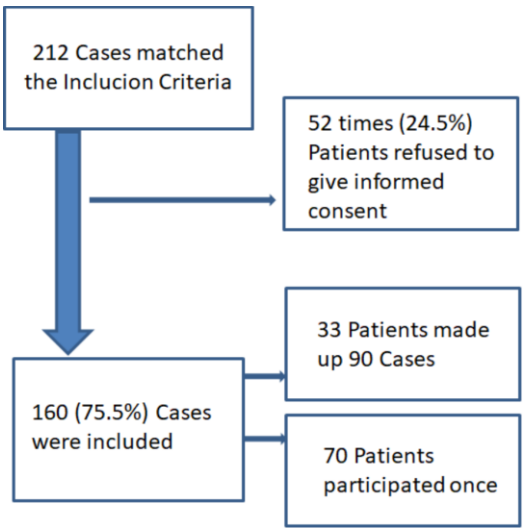

Figure 2: Drop-out rate of study population and the recurrent participation pattern of patients. This defines the difference between cases and actual patients

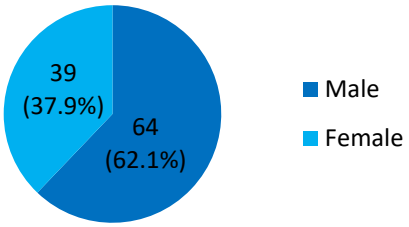

Figure 3: Sex distribution of study population

The mean age was 55 years; the youngest participant was 20 years old and the oldest was 81 years old. The underlying diseases of the participants were diverse and comprised nearly all fields of the hematology specialty. For more details, see Table 10.

Underlying diseases	Number
Acute Myeloid Leukemia, all Cases	31
AML, Type M0	10
AML, Type M1	4
AML, Type M2	1
AML, Type M3	3
AML, Type M4	8
AML, Type M5	4
AML, Type M6	1
Multiple myeloma, all cases	17
IgG kappa	8
IgA kappa	8
Secondary AML	11
Chronic lymphocyte leukemia, here B-cell Line	7
Myelodysplastic syndrome	7
Severe aplastic anemia	4
Diffuse large B-non-Hodgkin lymphoma	3
Diffuse large B-non-Hodgkin lymphoma, CNS lymphoma	3
Others	3
Follicular lymphoma	2
Mantle cell lymphoma	2
Burkitt lymphoma	2
Hairy cell leukemia	2
Acute lymphoblastic leukemia, here: Pro-B-ALL	2
Polycythemia vera	1
Primary myelofibrosis	1
Hodgkin lymphoma	1
Pro-T-cell lymphoblastic lymphoma	1
Plasma cell leukemia	1
Primary mediastinal large B-cell lymphoma	1
Mycosis fungoides	1
Anaplastic large cell lymphoma	1
Total	103

Table 7: Underlying diseases in study population The evaluation was performed per patient. AML=Acute Myeloid Leukemia, ALL=Acute Lymphoid Leukemia, CNS=Central Nervous System

The reasons for admission were categorized into four main groups: (I) evaluation and initiation of chemotherapy, (II) continuation of chemotherapy, (III) HSCT, and (IV) treatment of an infection. For detailed information per case, see Table 8.

Reasons for admission	Total
Allogeneic stem cell transplantation	30
Autologous stem cell transplantation	19
Diagnosis and therapy of acute GVHD	18
Therapy AML, induction I or II	16
Therapy AML, consolidation	12
Infection with fever	11
Therapy acute pneumonia	10
Evaluation underlying disease	9
Therapy AML, relapse	5
Therapy ALL/Burkitt Lymphoma, Hölzer protocol	7
Diagnosis and therapy of chronic GVHD	6
Therapy relapse lymphoma	4
Others	4
Neutropenic fever	3
Neurological symptoms	2
Therapy chronic lymphoid leukemia	2
Therapy lymphoma	1
Harvesting of stem cells	1
Total	160

Table 8: Reasons for admission evaluated per case AML=Acute Myeloid Leukemia, ALL=Acute Lymphoid Leukemia, GVHD=Graft versus Host Disease

All participants experienced 140 episodes of bone marrow depressions, of which febrile leukopenia occurred in 18 episodes. In total, participants suffered from 124 episodes of neutropenia, of which they developed neutropenic fever in 88 episodes. Ten participants developed a systemic inflammatory response syndrome or sepsis.

	Median	Mean	Min.	Max.	SD
Duration of hospitalization	27	32.51	5	122	22.2
Duration of neutropenia	12	14.24	1	84	10.97
Duration febrile neutropenia	3	5.06	1	26	5.83
Duration of BMS	19	25.2	1	412	37.24
Duration febrile leukopenia	5.5	6.72	1	18	5.52
Duration of SIRS/sepsis	4.5	6.8	1	30	8.54

Table 9: Time periods Min.=Minimum, Max.=Maximum, BMS=Bone Marrow Suppression, SD=Standard Deviation, SIRS=Systemic Inflammatory Response Syndrome. Durations is depicted in days

Forty-five participants died during or after the trial. Unfortunately, autopsies were performed on only 5 participants. Fungus-like fibroblastic proliferation into the alveoli was described in 1 participant. This participant had also been colonized by *Candida albicans* in his respiratory tract (positive sputum culture).

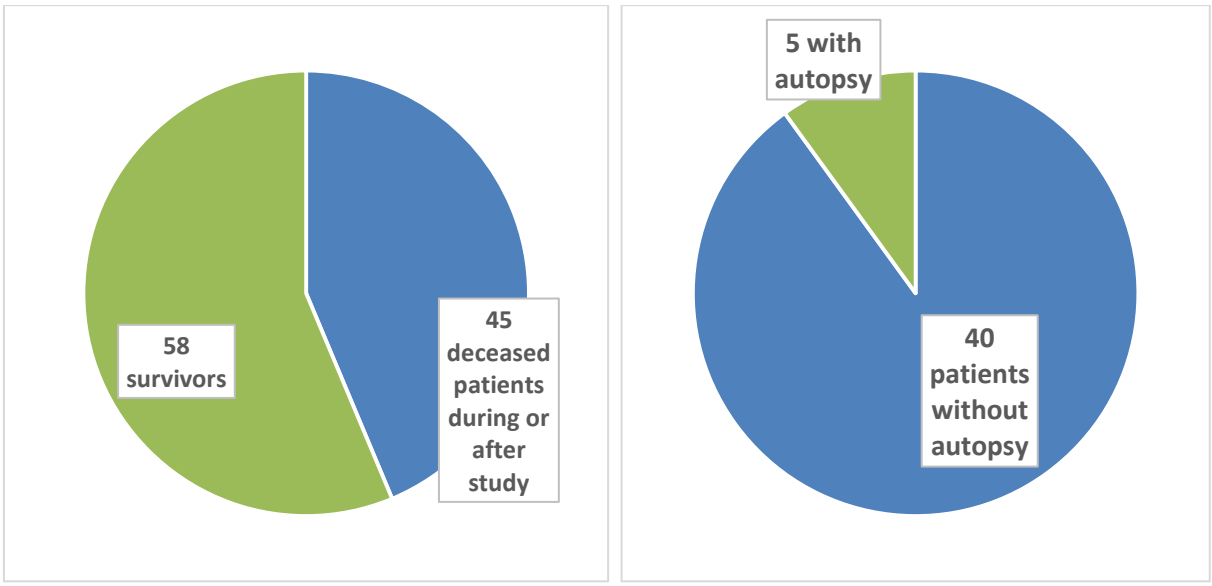

Figure 4: Death rate within study population and number of autopsies

6.1.2 Description of host factors and risk factors

Of the 160 cases, 131 (81.88%) cases and 156 (97.5%) cases matched the *Aspergillus* host factors and the risk factors invasive candidiasis, respectively.

6.1.2.1 Host factors of invasive aspergillosis

Aspergillus host factors	Numbers
(1) Neutropenia ($0.5x10^9$/L) >10 days	79
(2) T-cell suppressor or nucleoside analogues within the last 90 days	50
(3) Allogeneic stem cell transplant during trial	30
(4) Corticosteroid higher than 0.3mg/kg/day for >21 days	15
(5) Inherited immune deficiency, such as CGD	0
Number of Participants who matched these criteria	131

Table 10: Host factors for invasive pulmonary infections CGD=chronic granulomatous diseases

(1) Neutropenia was present in 118 cases, but in only 79 cases it lasted for more than 10 days, and in 54 cases for more than 14 days. In 52 cases, the participants suffered from relapsing neutropenia.

(2) In 50 cases, T-cell suppressants, other than corticosteroids, were administered during the trial. Cyclosporine A, mycophenolate, and anti-thymoglobulin were used and administered as monotherapy or in combination mainly due to the following reasons: (I) as part of a conditioning schedule, (II) to avoid a graft rejection, (III) to avoid GVHD, or (IV) for the therapy for severe aplastic anemia.

(3) Patients after allogeneic stem cell transplant bare a special risk for IA/IPA for approximately 100 days. Figure 5 depictures the distribution of HSCT before and during study. Altogether, 80 HCST took place, of what 49 ones (30 allogeneic HSCT, 19 autologous HCST) happened during the study and 31 ones (22 allogeneic HSCT, 19 autologous, 1 cord blood HSCT) before. In the 100 day range directly after transplant were eleven allogeneic HSCT patients and one with autologous HSCT.

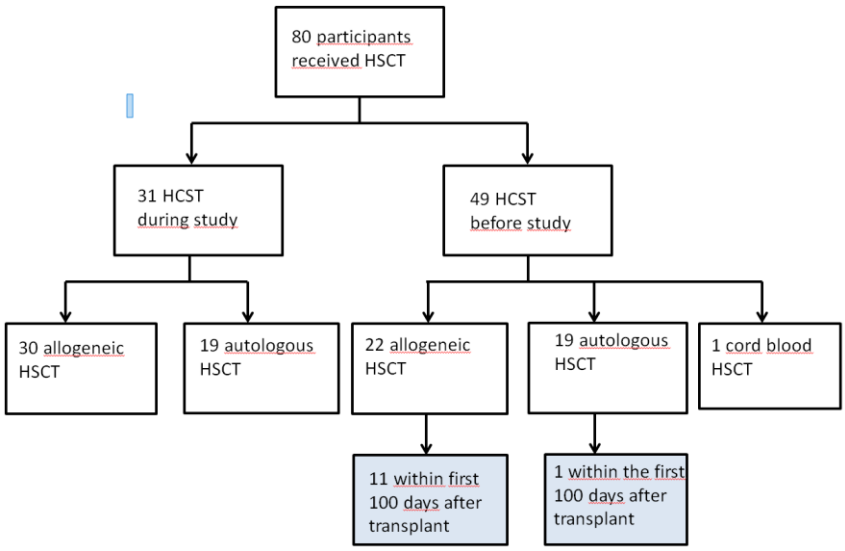

Figure 5: Demographic distribution of hematopoietic stem cell transplant recipients

HSCT=Hematopoietic Stem Cell Transplant

(4) Of the 160 cases, 82 received corticosteroids therapy due to the following reasons: (I) GVHD, (II) as a part of chemotherapy schedule, and (III) prophylaxis for expected anaphylactic reactions to monoclonal antibodies. Of the 82 cases, 15 received the high-dose corticosteroids therapy (defined as ≥0.3mg/kg/day administered for ≥21 days).

(5) No participant suffered from chronic granulomatous disease.

6.1.2.2 Risk factors of invasive candidiasis

(1) Of the 160 cases, 150 received a broad-spectrum antibiotic as prophylaxis or as a first-line therapy. A change to other antibiotics due to treatment failure or therapy adjustment based on susceptibility test results of microorganisms was not an issue.

Candida risk factors	Numbers
(1) Therapy with broad-spectrum antibiotics	154
(2) Chemotherapy	122
(3) Neutropenia*	118
(4) Indwelling devices such as CVC, urinary catheter	117
(5) High-dose chemotherapy	106
(6) Therapy with corticosteroids	81
(7) Neutropenia ($0.5x10^9$/L) >10 days	79
(8) Neutropenia ($0.5x10^9$/L) >14 days	54
(9) Fungal colonization	34
(10) Stay at intensive care unit	9
(11) Total parenteral nutrition	7
(12) Diabetes mellitus 2	5
(13) HIV/AIDS**	1
(14) Recent surgery in the GI tract	0
(15) Number of matched criteria	156

Table 11: Risk factors for *Candida* CVC=Central Venous Catheter *Neutropenia of one single day also considered; ** One patient with HIV participated, who was treated well with HAART (Highly active Anti-retroviral Therapy) schedule

(2, 5) Of the 160 cases, 122 received chemotherapy. Of the 122 cases with chemotherapy, 106 received high-dose chemotherapy due to the following reasons: (I) as a part of conditioning before HSCT, (II) induction/re-induction therapy of AML, (III) consolidation therapy of AML or (IV) therapy of relapsing disease including patients with lymphoma.

(3, 7, 8) Regarding the feature “neutropenia”, see the results of “*Aspergillus* host factors”.

(4) In 126 cases, the participants received a CVC during their stay at the hospital. The median duration was 28 days (mean: 31.52 days) with a minimum of 3 days and a maximum of 89 days. Furthermore, 8 participants received a Foley catheter for the following reasons: (I) fluid equilibration, (II) support during phases of severe weakness, or (III) mental issues. The median duration was 10 days (mean: 68.57 days) with a minimum of 3 days and a maximum of 422 days.

(6) Regarding the feature “therapy with corticosteroids”, we refer to the results of “*Aspergillus* host factors”.

(9) *Candida* colonization is an important source of an invasive candidiasis in immunosuppressed patients. In 34 cases, colonization was observed, and the participants were affected in single or multiple manners. In 8 of the 34 cases, *Candida* colonization in the participants' BAL fluid specimens was detected by microscopic cytology or by plate cultures made from the BAL fluid samples (4x *Candida albicans* and 4x *Candida glabrata*). However, it was not possible to exclude a contamination from the upper respiratory tract. In 16 of 34 cases, *Candida* colonization was detected by a swab and subsequent microscopic workup (of the anus, mouth, sputum, or wounds): 8x *Candida albicans*, 2x *Candida glabrata*, 2x *Candida albicans* et *Candida glabrata*, 2x the subspecies was not recognizable and declared as yeast, 1x *Candida krusei* and 1x *Alternaria alternate* et *Geotrichum capitatum*. In 15 of the 34 cases, *Candida* mucositis occurred. The median duration was 7 days (mean: 7.27 days) with a minimum of 2 days and a maximum of 16 days.

(10) Nine patients required admittance to the ICU due to acute pneumonia, single organ failure, or sepsis. The median duration of the stay was 6 days (mean: 10.67 days) with a minimum of 1 day and a maximum of 31 days.

(11) In 35 cases, parenteral nutrition was required. The median duration was 13 days (mean: 21.35 days) with a minimum of 1 day and a maximum of 108 days. Finally, in 7 cases, total parenteral nutrition was initiated with a median duration of 15 days (mean: 70.71 days) with a minimum of 4 days and a maximum of 412 days. The reasons for this initiation were due to the following reasons: (I) support during the phase of HSCT, (II) painful *Candida* thrush, (III) mental issues, and (IV) end-stage disease or rarer reasons that hindered the oral intake of food.

(12) Five patients suffered from co-existing Diabetes mellitus 2.

(13) No patient suffered from idiopathic low CD4+ T-cell counts. One patient suffered from HIV (and Burkitt Lymphoma) but was virologic suppressed by highly active antiretroviral therapy (HAART).

(14) Surgery was performed shortly before the start of the trial or during the trial on a total of 11 participants. Each procedure was performed once: computed tomography -guided biopsy, tooth extraction, Port-a-Cath system implantation or explanation, lymph node extirpation, Omaya reservoir application, abscess drainage, or pleuracentesis. No abdominal surgery involving the GI tract was performed, and no participant suffered from major trauma or burns.

(15) All participants received radiated blood products; therefore, these products did not constitute the source of *Candida* inoculation and were not considered in this trial.

6.2 Microbiological work-up results of study population

In this trial, 29 BAL procedures were performed to obtain samples. The reasons for these procedures were: (I) fever, (II) clinical evidence of pneumonia, or (III) newly developed signs of infection in chest X-ray or scanning. The microbiological workup included cytology via microscopic and staining procedures as well as plate cultures made from BAL fluid samples. Blood cultures were drawn 106 times due to clinical evidence such as fever or leukocytosis without the use of Granulocyte Colony-Stimulating (GCS) Factor.

Name of microorganisms	Number	Name of microorganisms	Number
Mixed flora*	8	*Staphylococcus aureus*	1
Klebsiella spp.	2	*Serratia* spp.	1
Enterococcus spp.	2	*Lactobacillus* spp.	1
Enterobacter cloacae	1	*Candida albicans*	1
MRSA	1	*Candida glabrata*	1
Bifidobacterium	1	No evidence of microorganisms	16
Acinetobacter spp.	1		

Table 12: Microbiological results of BAL fluid smears Mixed Flora*=Mixed flora with apathogenic *Neisseria* and *Streptococcus*, MRSA=Methicillin-resistant *Staphylococcus aureus*, BAL=Bronchoalveolar Lavage, spp.=Species

Name of microorganisms	Number	Name of microorganism	Number
Candida glabrata	4	*Microbacterium* spp.	1
Candida albicans	4	*Staphylococcus haemolyticus*	1
Enterococcus faecialis	4	*Staphylococcus epidermidis*	1
Lactobacillus spp.	4	*Micrococcus* spp.	1
Streptococcus viridans group	4	*Serratia marcescens*	1
Prevotella melaninogenica	4	*Staphylococcus aureus*	1
Klebsiella oxytoca	4	*Haemophilus parainfluenzae*	1
Enterococcus faecium	3	MRSA	1
No evidence of Microorganisms	3	*Rothia mucilaginosa*	2

Table 13: Plate culture results of BAL fluid samples MRSA=Methicillin-resistant *Staphylococcus aureus*, BAL=Bronchoalveolar Lavage, spp.=Species

Name of microorganisms	Number	Name of microorganisms	Number
Staphylococcus epidermidis	20	*Stenotrophomonas maltophilia*	1
Enterococcus spp.	7	*Staphylococcus xylosus*	1
Staphylococcus hominis	5	*Streptococcus salivarius*	1
Escherichia coli	4	*Streptococcus* of group A	1
Pseudomonas spp.	3	MRSA	1
Escherichia coli, ESBL	2	*Klebsiella pneumoniae*	1
Candida albicans	3	*Klebsiella oxytoca*	1
Sphingomonas koreensis	1	*Proteus* spp.	1
Staphylococcus capitis	1	*Acinetobacter* spp.	1
Staphylococcus auricularis	1	*Enterobacter cloacae*	1
Streptococcus sanguinis	1	*Total microorganisms*	58
Streptococcus oralis	1		

Table 14: Results of blood cultures ESBL=Extended Spectrum of Beta-Lactamase *Escherichia coli*, MRSA=Methicillin-resistant *Staphylococcus aureus*, spp.=Species

CVC tips of 2 patients were contaminated with *Staphylococcus epidermidis* only, another 2 with *Staphylococcus epidermidis* and *Stenotrophomonas maltophilia*, and another 1 with *Candida albicans*.

6.3 Overview of the results of medical imaging in study population

Chest CT scans were performed in 81 cases and in 35 of these cases were suspicious for fungal infections (43.21%). The signs of infection were as follows: (I) alveolar infiltrates, (II) interstitial infiltrates, (III) nodules (among others the halo sign), (IV) masses, and (V) cavitation's (air-crescent sign). Some scans showed abscesses, adenopathy or pleural effusion only and were also classified as suspicious scans, although they are not common signs of IPA, but of invasive candidiasis, mucormycosis, pneumocystis pneumonia, or cryptococcosis.

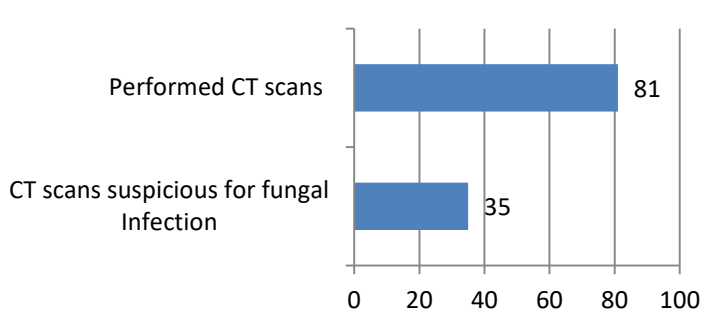

Figure 6: Performed CT scans and the amount suspicious for fungal infection. The X-axis presents the amount in natural numbers

6.4 Frequency of invasive fungal infections

In this chapter, we give an overview of the IFIs, which occurred, in our study.

6.4.1 Invasive pulmonary aspergillosis and invasive aspergillosis

Of the 160 cases, 11 (6.88%) cases were classified as probable IPA and 22 (13.75%) cases as possible IPA due to the revised EORTC/MSG guidelines. No proven IPA or IA occurred in this study population. The remaining cases were classified as not having IPA. Of these 127 cases, 6 (4.72%) cases had clinical signs and symptoms compatible to an IPA; they had, for instance, neutropenic fever and had positive GM values in serum , but for practical reasons, had only chest X-rays performed. The practical reasons were as follows: (I) severe neutropenia (patient received an X-ray in bed), (II) severely ill patients were spared (and received an X-ray in bed), (III) chest scan beforehand (without signs of pneumonia) that should not be repeated, and (IV) bacterial or viral pneumonia was expected as the first-line diagnosis. This subgroup that could not be classified properly as possible or probable IPA cases was excluded from further evaluation.

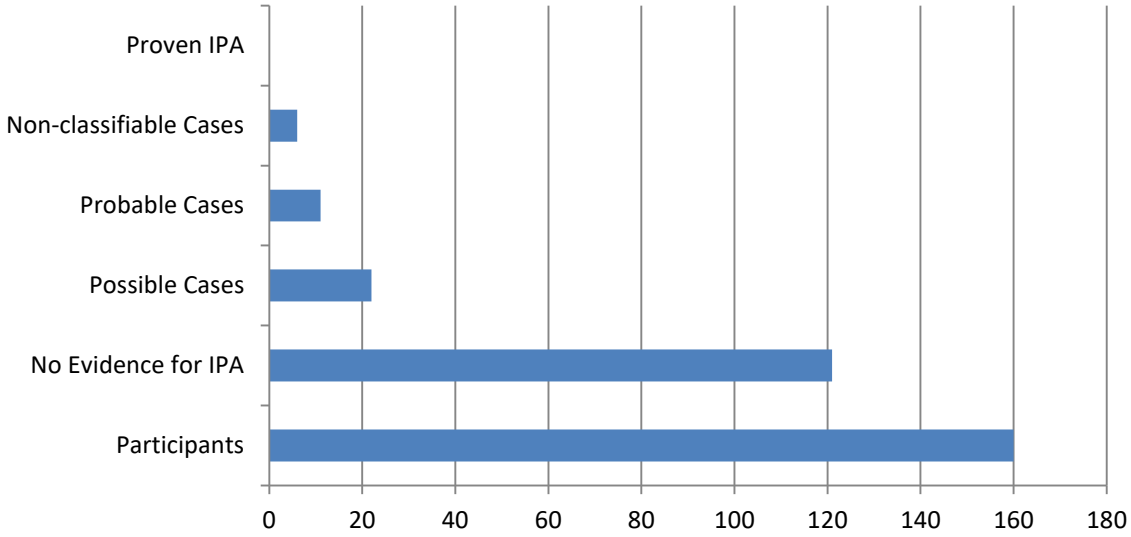

Figure 7: Numbers of classification per the modified EORTC/MSG criteria The X-axis presents the amount of cases in natural numbers. "Non-classifiable Cases" are participants who show signs and symptoms of an IPA and have positive GM assay test results in serum and/or BAL fluid samples but received for practical reasons an X-ray of the chest but no CT scan. IPA=Invasive Pulmonary Infection, GM=Galactomannan

For the treatment of the probable IPA cases, caspofungin (6x) or voriconazole (3x) were preferred. Posaconazole was used in one case as well as anidulafungin. If the treatment was adjusted due to ongoing symptoms voriconazole (6x) was chosen as second line therapy. Third line therapy was LAB (2x). In two cases a posaconazole prophylaxis was performed so that the IPA is accurately defined as breakthrough invasive aspergillosis. In both cases a non azole-active drug was chosen for therapy thus caspofungin. Reasons for therapy switch are ongoing fever or other signs and symptoms of an invasive aspergillosis.

The shortest first line therapy lasted one day (and was adjusted to voriconazole afterward); the longest therapy lasted 16 days, the mean was 6.72 days. The second line therapy lasted four days at minimum and 17 days at maximum. The mean was 9 days. And the third line therapy showed a maximum duration of 13 days, a minimum duration of one day, and a mean of 5.67 days.

Six patients died over the course of time. The reasons for death were not clearly definable due to a lack of autopsies. However, the survivors with probable IPA all received mold-active antifungal therapy for a mean of 12.67 days (maximum: 19 days, minimum: 6 days).

6.4.2 Invasive candidiasis

In this trial, 3/160 (1.88%) participants had candidemia proven by blood culture. The blood cultures had been routinely drawn due to fever and other symptoms of infections.

The first line treatment for invasive candidiasis is caspofungin. No switch to another substance took place. The treatment lasted shortest for 13 days and longest for 43 days. The mean was 24 days. All patients with proven candidemia died over the course of time.

6.5 Analysis of the biomarkers

In this chapter, we present the statistical analyses of the biomarkers in the varying specimen.

6.5.1 Galactomannan assay test results in serum samples

Here we analyzed the GM assay in serum samples and compared the test results to the co-occurred test results in BAL fluid samples.

Parts of this data were published in *Duettmann Wiebke, Koidl Christoph, Prochazka (formerly Troppan) Katharina, Hoenigl (formerly Seeber) Katharina, Buzina Walter, Wölfler Albert, Rabensteiner (formerly Wagner) Jasmin, Krause Robert, Hoenigl Martin* **Serum and urine galactomannan testing for screening in patients with hematological malignancies.** Med Mycol. 2014 Aug;52(6):647-52. doi: 10.1093/mmy/myu019. Epub 2014 Jun 17. Data, which were partly evaluated in this publication, were also included into the whole data set and used within the thesis with permission of the Journals.

6.5.1.1 Descriptive analyses of entire sample set

We retrieved 1109 serum samples from the 160 cases from which we received GM assay test results during the study period. For more information, see Table 15 and Figure 8. Samples were considered as positive at ≥ 0.5ODI.

GM serum [ODI]	
N	1109
Mean	0.17
Median	0.09
SD	0.27
Minimum	0.00
Maximum	4.50

Table 15: Descriptive statistic of test results of the entire galactomannan assay test results in serum samples SD=Standard Deviation

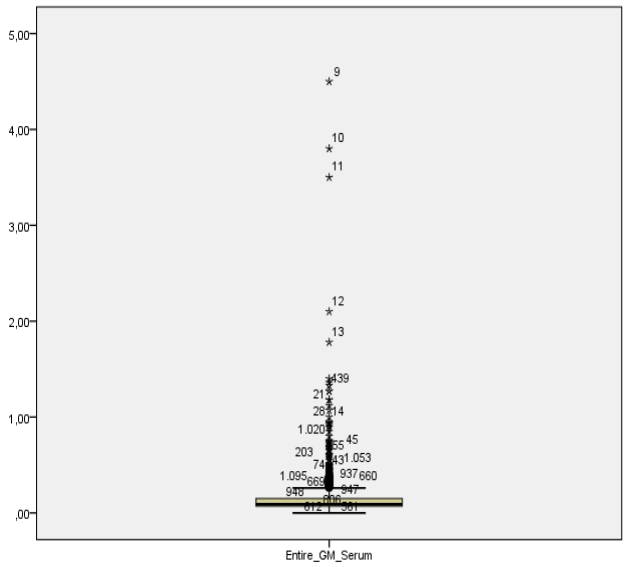

Figure 8: Box plot of entire galactomannan test results in serum samples

6.5.1.2 Descriptive analysis of GM assay test results in aspergillus subgroup

For this analysis, we used a purified subgroup, which included only the samples from participants who matched the *Aspergillus* host factors and who were negative for proven invasive candidiasis. Therefore, we had to exclude 24 cases, which did not match *Aspergillus* host factors, as well as 3 cases with proven candidemia. For further evaluation, we chose a case-based approach (and not a sample-based approach). Finally, we had 133 cases in the purified *Aspergillus* subgroup.

In [ODI]	No IPA	Possible IPA	Probable IPA
N	757	213	94
Mean	0.14	0.11	0.14
Median	0.09	0.08	0.11
SD	0.13	0.09	0.77
Minimum	0.00	0.00	0.00
Maximum	1.27	0.45	4.50

Table 16: Descriptive statistic of test results of the galactomannan assay test results in *Aspergillus* subgroups SD=Standard Deviation, IPA=Invasive Pulmonary Aspergillosis

In Table 16, we show the frequencies of the GM assay test results of the subgroups classified according to the modified EORTC/MSG guidelines. Afterward, we highlight the data with help of box plots (Figure 9) as follows: Entire sample set (Entire_GM), cases without signs for an IPA (No_IPA), cases classified as possible IPA (Possible_IPA), and the GM assay test results of the probable cases (Probable_IPA).

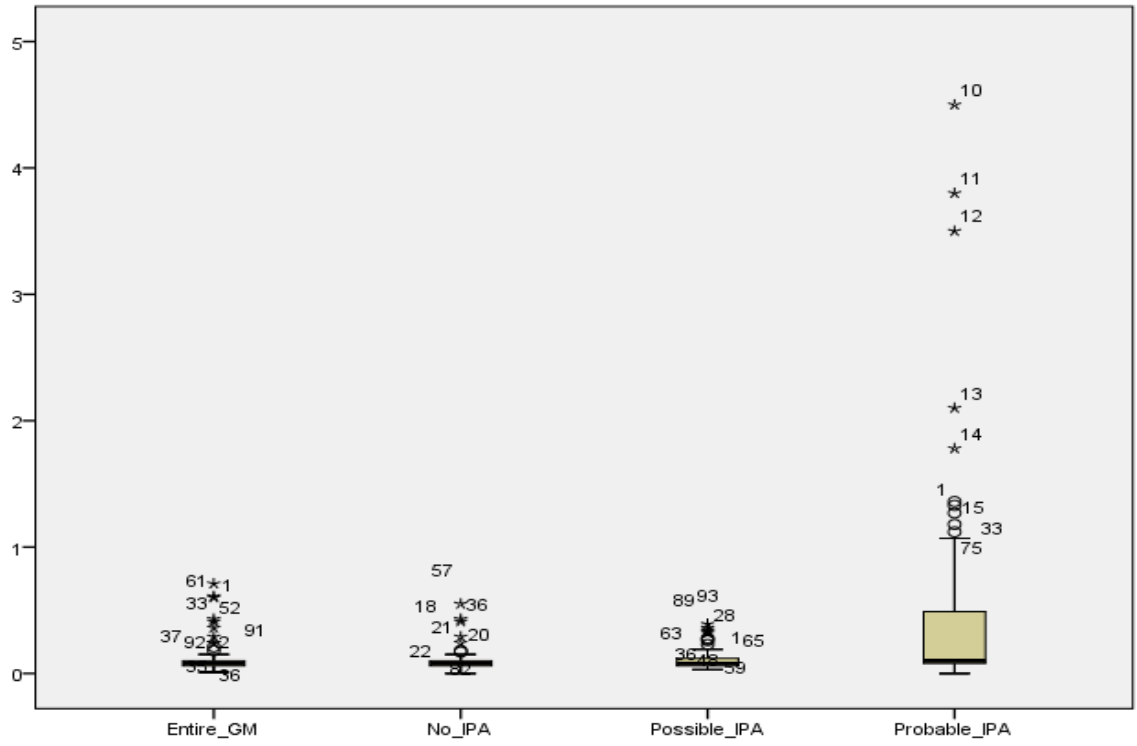

Figure 9: Box plots of galactomannan test results in *Aspergillus* subgroups GM=Galactomannan, IPA=Invasive Pulmonary Aspergillosis

6.5.1.3 Performance of galactomannan assay test results

As mentioned above, we used a cutoff of 0.5ODI to classify a sample as positive. In 19/133 (14.29%) cases the GM assay test results were positive and in 12/19 (63.16%) cases, the test results were classified as false-positive due to negative GM assay test results in BAL fluid samples and lack of signs and symptoms. No reasons for the false positivity could be found, however, the simultaneous application of antibiotics (such as piperacillin/tazobactam or amoxicillin) was ruled out. Altogether 39 patients received piperacillin/tazobactam and none of those had co-occurring positive GM assay test results. The remaining 7/19 (36.84%) cases were classified as true-positive compared to clinical condition, medical imaging, and/or positive GM assay test results in BAL fluids of the same case. In 114/133 (85.71%) cases the GM assay test results remained negative. We classified 4/113 (3.54%) as false-negative due to positive GM assay test results in BAL fluid samples in a patient with signs and symptoms of an IPA. The remaining 110/113 (97.35%) cases were classified as true-negative.

Aspergillus cases	FP	TP	FN	TN
Probable IPA	0	7	4	0
Possible IPA	0	0	0	23
No IPA	12	0	0	87

Table 17: Test results of galactomannan assay We evaluated the samples per cases. For analysis, the participants were classified according to the modified EORTC/MSG criteria in possible, probable or proven cases. Participants who did not meet the Aspergillus host factors and who suffered from proven invasive candidemia were excluded from further evaluation. FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, IPA=Invasive Pulmonary Aspergillosis

Aspergillus cases	Sensitivity	Specificity	PPV	NPV
Probable IPA	63.6%	N/A	100%	N/A
Possible IPA	N/A	100%	N/A	100%
No IPA	N/A	87.9%	N/A	100%
Summary	63.6%	87.9%	36.8%	95.6%

Table 18: Performance of galactomannan assay in serum samples evaluated per cases PPV=Positive Predictive Value, NPV=Negative Predictive Value, N/A=Not Available

We evaluated the GM assay in serum samples for two consecutive positive test results in order to increase the sensitivity and specificity. We found only eight cases. Table 19 and 20 show the test results in detail:

Aspergillus cases	FP	TP	FN	TN
Probable IPA	0	2	0	0
Possible IPA	0	0	0	1
No IPA	5	0	0	0

Table 19: Galactomannan assays in serum samples evaluated for two consecutive positive test results FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, IPA=Invasive Pulmonary Aspergillosis

Aspergillus cases	Sensitivity	Specificity	PPV	NPV
Probable IPA	100%	N/A	100%	N/A
Possible IPA	N/A	100%	N/A	100%
No IPA	N/A	0%	N/A	0%
Summary	100%	16.7%	28.6%	100%

Table 20: Performance of galactomannan assays in serum samples evaluated for two consecutive positive test results IPA=Invasive Pulmonary Aspergillosis, N/A=Not Available, PPV=Positive Predictive Value, NPV=Negative Predictive Value

Furthermore, we evaluated our cases for simultaneous application of anti-mold active medications. Voriconazole, posaconazole, itraconazole, LAB and echinocandin (caspofungin, micafungin) are anti-mold active whereas fluconazole is not. We found 76 cases, which were treated with an antifungal medication when doctors in charge ordered a CT scan of the chest in order to find reasons for a sudden onset of worsening health conditions. However, no patient received fluconazole.

Aspergillus cases	FP	TP	FN	TN
Caspofungin	2	1	0	9
Probable IPA	0	1	0	0
Possible IPA	1	0	0	1
No IPA	1	0	0	8
Micafungin	0	0	0	4
Probable IPA	0	0	0	0
Possible IPA	0	0	0	1
No IPA	0	0	0	3
Voriconazole	2	0	0	16
Probable IPA	0	0	0	0
Possible IPA	1	0	0	6
No IPA	1	0	0	10
Posaconazole	3	1	1	33
Probable IPA	0	1	1	0
Possible IPA	0	0	0	9
No IPA	3	0	0	24
Itraconazole	0	0	0	1
Probable IPA	0	0	0	0
Possible IPA	0	0	0	0
No IPA	0	0	0	1
LAB	0	0	0	3
Probable IPA	0	0	0	0
Possible IPA	0	0	0	1
No IPA	0	0	0	2

Table 21: Test results of galactomannan in serum samples regarding anti-mold active agents FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, IPA=Invasive Pulmonary Aspergillosis, LAB=Liposomal Amphotericin B

Due to low sample size, we completed the performance analyses of the biomarkers only regarding the antifungal drugs and refrained from doing so with the subgroups (classification as probable, possible or no IPA case).

6.5.2 Galactomannan assay test results in BAL fluid samples

Bronchoscopies and BALs were performed 24 times within the *Aspergillus* subgroup due to severe worsening of health conditions and newly developed signs in CT scans (sometimes in X-rays of the chest). The *Aspergillus* subgroup for BAL fluid samples was defined the same as for serum samples (all participants who matched the *Aspergillus* host factors and those who did not have a proven invasive candidiasis). However, in two patients two episodes of neutropenic fever occurred during one stay at the hospital, so that two chest CT scans and two bronchoscopies with BALs were performed. We evaluated both GM assay test results in BAL fluid samples, but it must be kept in mind that the focus here is on 20 cases (and 22 samples). In addition, unfortunately, we have only the information “negative for GM assay test result” for two more BAL fluid samples. The GM assay test results in BAL fluid samples are shown in Table 22.

6.5.2.1 Descriptive Analyses of entire sample set

ln [ODI]	GM BAL
N	22
Mean	0.42
Median	0.16
SD	0.64
Minimum	0.05
Maximum	2.8

Table 22: Descriptive statistic of test results of galactomannan assays test results in all BAL fluid samples GM=Galactomannan, SD=Standard Deviation, BAL= Bronchoalveolar Lavage

6.5.2.2 Descriptive analysis of GM assay test results in Aspergillus subgroup

ln [ODI]	No IPA	Possible IPA	Probable IPA
N	8	9	7
Mean	0.09	0.16	1.02
Median	0.08	0.12	0.80
SD	0.05	0.14	0.90
Minimum	0.05	0.00	0.15
Maximum	0.2	0.43	2.8

Table 23: Descriptive statistic of test results of galactomannan assays test results in all BAL fluid samples of the *Aspergillus* subgroups GM=Galactomannan, SD=Standard Deviation, BAL= Bronchoalveolar Lavage, IPA=Invasive Pulmonary Aspergillosis

In Table 23, we show the GM assay test results in BAL fluid samples and in Table 24 the simultaneously drawn GM assay test results in serum samples. Due to dynamic changes of GM in blood, we also evaluated the entire serum samples of the 22 cases (who had 24 bronchoscopies) and not only the serum sample obtained on the same day as the BAL fluid sample (no sample-sample comparison, but sample-case comparison). The box plot of Figure 10 presents the GM assay test results of the BAL fluid samples of the subgroups No_IPA_BAL, Possible_IPA_BAL, and Probable_IPA_BAL. Finally, the box plots of Figure 11 present the direct comparison of data. Box plots were sorted as follows: Cases without IPA and the related GM assay test results in BAL fluid samples (box plot 1, No_IPA_BAL) compared to assay test results in serum (box plot 2, No_IPA_Serum). The third and fourth box plots represent the assay test results in cases classified as possible IPA (Possible_IPA_BAL, Possible_IPA_Serum). Finally, box plots five and six show the assay test results in probable cases (Probable_IPA BAL, Probable_IPA_Serum).

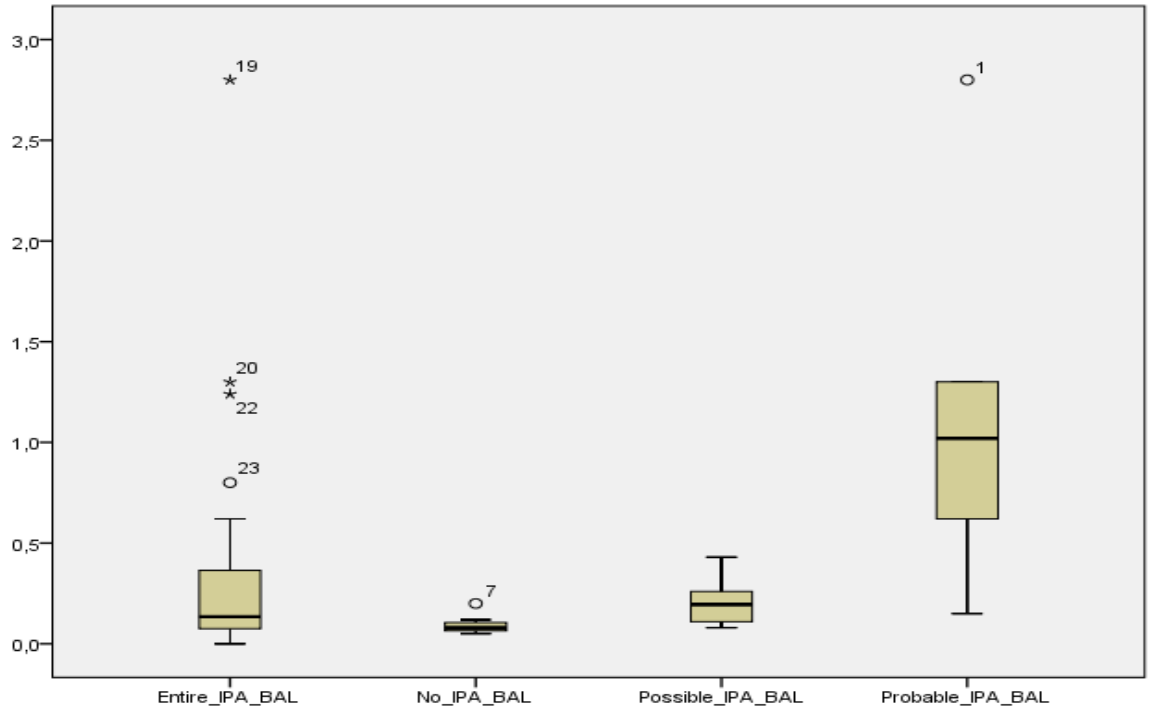

Figure 10: Box plots of galactomannan assay test results in all BAL fluid samples IPA=Invasive Pulmonic Aspergillosis, BAL=Bronchoalveolar Lavage

In [ODI]	No IPA	Possible IPA	Probable IPA
N	81	80	53
Mean	0.14	0.12	0.17
Median	0.10	0.85	0.09
SD	0.11	0.09	0.22
Minimum	0.01	0.04	0.00
Maximum	0.75	0.45	1.33

Table 24: Descriptive statistic of test results of galactomannan test results of the simultaneously with BAL drawn serum samples GM=Galactomannan, SD=Standard Deviation, BAL= Bronchoalveolar Lavage

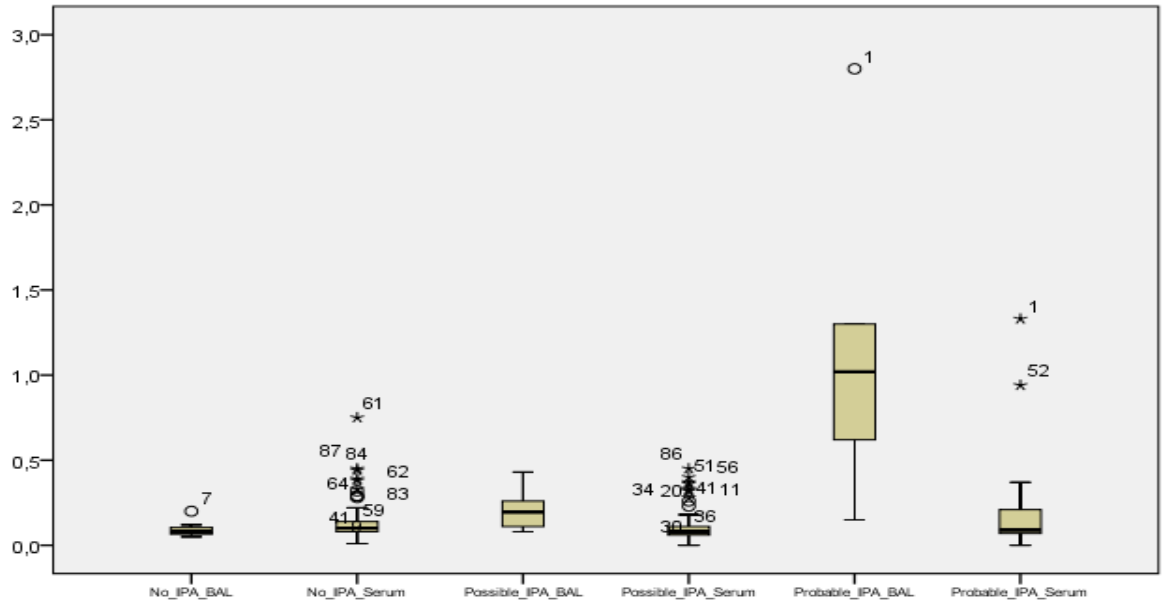

Figure 11: Box plots of distribution of galactomannan assays test results in all BAL fluid samples and in the *Aspergillus* subgroups IPA=Invasive Pulmonary Aspergillosis, BAL= Bronchoalveolar Lavage

6.5.2.3 Performance of galactomannan assay test results in BAL fluid samples

The GM assay was performed 24 times on BAL fluid samples. In 5/24 (20.83%) samples the GM assay in BAL fluid fluids was positive and no sample was classified as false-positive. The remaining 19/24 (79.17%) samples stayed negative from which 1/19 (5.26%) was classified as false-negative due to a simultaneous suspicious lung CT scan and the fact that the respective serum samples showed a positive GM assay test result. However, no reason for false positivity was found (*e.g.* simultaneous application of piperacillin/tazobactam or amoxicillin). The remaining 18/19 (94.74%) were classified as true-negative.

Aspergillus cases	FP	TP	FN	TN
Probable IPA	0	5	1	0
Possible IPA	0	0	0	10
No IPA	0	0	0	8

Table 25: Test results of galactomannan assays in BAL fluid samples IPA=Invasive Pulmonary Aspergillosis, FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative

Aspergillus cases	Sensitivity	Specificity	PPV	NPV
Probable IPA	83.3%	N/A	100%	N/A
Possible IPA	N/A	100%	N/A	100%
No IPA	N/A	100%	N/A	100%
Summary	83.3%	100%	100%	94.7%

Table 26: Performance of galactomannan assay in BAL fluid samples IPA=Invasive Pulmonary Aspergillosis, PPV=Positive Predictive Value, NPV=Negative Predictive Value, N/A=Not Ascertainable

6.5.3 Galactomannan assay test results in urine samples

Parts of this data were published in *Duettmann Wiebke, Koidl Christoph, Prochazka (formerly Troppan) Katharina, Hoenigl (formerly Seeber) Katharina, Buzina Walter, Wölfler Albert, Rabensteiner (formerly Wagner) Jasmin, Krause Robert, Hoenigl Martin* **Serum and urine galactomannan testing for screening in patients with hematological malignancies.** Med Mycol. 2014 Aug;52(6):647-52. doi: 10.1093/mmy/myu019. Epub 2014 Jun 17. Data, which were partly evaluated in this publication, were also included into the whole data set and used within the thesis with permission of the Journals.

ln [ODI]	GM urine
N	202
Mean	0.02
Median	0.00
SD	0.07
Minimum	0.00
Maximum	1.50

Table 27: Descriptive statistic of test results of galactomannan assay test results in urine samples SD=Standard Deviation

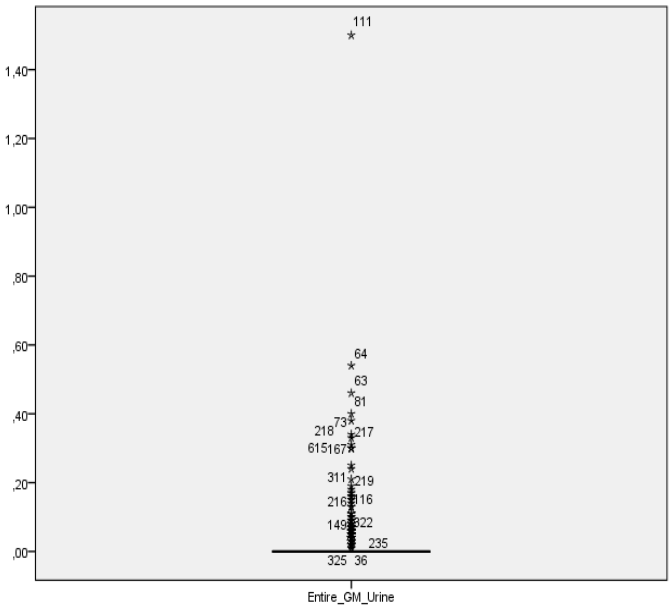

Figure 12: Box plot of galactomannan assays test results in all urine samples

In the *Aspergillus* subgroup, the GM assay was performed on 201 urine samples, which represented 67 cases. We evaluated the data only case-based and not sample-to-sample based.

6.5.3.1 Descriptive analysis of galactomannan assay test results

In table 28, we present the descriptive evaluations of the GM assay test results in urine samples, which are sorted according to the revised EORTC/MSG guidelines as we did for the previous sample evaluations.

ln [ODI]	No IPA	Possible IPA	Probable IPA
N	149	36	16
Mean	0.14	0.66	0.13
Median	0.05	0.05	0.09
SD	0.14	0.06	0.12
Minimum	0.01	0.02	0.01
Maximum	1.5	0.30	0.38

Table 28: Descriptive statistic of test results of galactomannan assays test results in all urine samples of the *Aspergillus* subgroups GM=Galactomannan, SD=Standard Deviation, IPA=Invasive Pulmonary Aspergillosis

In the box plots presented in Figure 13, the assay test results are sorted as follows: The first three box plots show the assay test results in cases without any signs and symptoms of IPA (No_IPA_Urine, No_IPA_Urine_Serum, No_IPA_Urine_BAL). The next box plots represent the GM assay test results in possible cases in urine samples (box plot 4, Poss_IPA_Urine) compared to box plots 5 and 6, which represent test results of the same cases but in serum (Poss_IPA_Urine_Serum) as well as in BAL fluid samples (Poss_IPA_Urine_BAL). The last three box plots (7, 8, 9) show the GM assay test results in the probable subgroup in urine samples (7, Prob_Urine_BAL) compared to the probable serum cases (8, Prob_IPA_Urine_Serum) and BAL fluid samples (9, Prob_IPA_Urine_BAL).

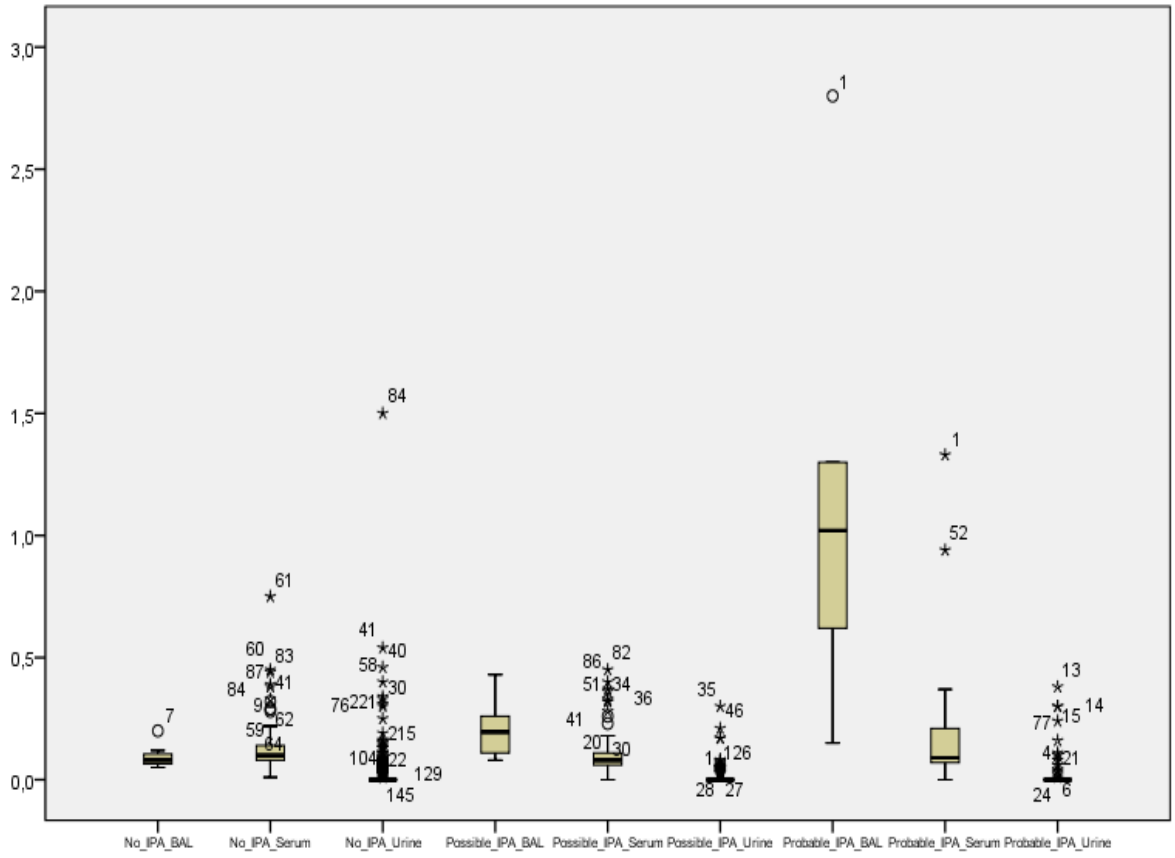

Figure 13: Box plots of galactomannan assays in urine samples and box plots of the corresponding serum samples IPA=Invasive Pulmonary Aspergillosis, BAL= Bronchoalveolar Lavage

6.5.3.2 Performance of galactomannan assay test results in urine samples

We used a non-confirmed cutoff of 0.1ODI to consider a sample as positive as we highlighted in the first interim report. We found 67 cases to evaluate, from which 23/67 (43.33%) cases were positive, but we classified 3/23 (13.04%) as true-positive and 20/23 (86.96%) as false-positive compared to the test results of GM assay test results in serum and BAL fluid samples and the health condition of the patient. No reason for false positivity was found. The remaining 44/67 (65.67%) cases remained negative, from which we classified 3/44 (6.82%) cases as false-negative. We could not find reasons for the false-negativity. Finally, 41/44 (93.18%) cases were true-negative.

Aspergillus cases	FP	TP	FN	TN
Probable IPA	0	3	3	0
Possible IPA	3	0	0	9
No IPA	17	0	0	32

Table 29: Test Results of galactomannan assays in urine samples FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, IPA=Invasive Pulmonary Aspergillosis

Aspergillus cases	Sensitivity	Specificity	PPV	NPV
Probable IPA	50%	N/A	100%	N/A
Possible IPA	N/A	75%	N/A	100%
No IPA	N/A	65.3%	N/A	100%
Summary	50%	61.5%	13%	91.4%

Table 30: Performance of galactomannan assay in urine samples PPV=Positive Predictive Value, NPV=Negative Predictive Value, IPA=Invasive Pulmonary Aspergillosis, N/A=Not Ascertainable

6.5.4 Aspergillus lateral flow device prototype test in BAL fluid samples

Altogether, the prototype *Aspergillus* LFD test was performed on 22 BAL fluid samples. In total, we had access to 24 samples, but there was insufficient material in two samples, therefore we could not perform the *Aspergillus* LFD prototype test on these. No test result was stronger than 1 (+) (Note: 3 (+++) is the achievable maximum), and the median was classified as 0 (−). We analyzed the samples in a sample-to-sample approach.

In 3/22 (9.09%) samples, the test results were mild positive (3x1 [+]). One-third of samples could not be classified with certainty (0-1), thus, it was excluded from further evaluation. All positive test results were classified as false-positive due to a negative GM assay test results in the same BAL fluid samples. However, the false-positive samples were also positive for the following microorganisms made from the culture of the BAL fluid: *Candida glabrata*, *Candida albicans*, and *Enterococcus faecium*. Additionally, *Legionella* PCR showed positivity in one sample. The 20/22 (90.91%) samples remained negative. We classified 6/22 (27.27%) as false-negative due to positive GM assay test results in the same BAL fluid sample or in the serum sample drawn on the same day. The remaining 14/22 (63.64%) samples were classified as true-positive.

Aspergillus cases	FP	TP	FN	TN
Probable IPA	0	0	6	0
Possible IPA	2	0	0	6
No IPA	0	0	0	8

Table 31: *Aspergillus* LFD prototype test results in BAL fluid samples FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, IPA=Invasive Pulmonary Aspergillosis

Aspergillus cases	Sensitivity	Specificity	PPV	NPV
Probable IPA	0%	N/A	0%	N/A
Possible IPA	N/A	75%	N/A	100%
No IPA	N/A	100%	N/A	100%
Summary	0%	87.5%	0%	70%

Table 32: Performance of *Aspergillus* prototype LFD test in BAL fluid samples PPV=Positive Predictive Value, NPV=Negative Predictive Value, IPA=Invasive Pulmonary Aspergillosis, N/A=Not Ascertainable

6.5.5 1,3-β-D-glucan assay test results in serum samples

In this chapter, we highlight the statistical evaluation of the BDG assay in serum samples. In total, we performed the BDG assay on 1079 serum samples.

6.5.5.1 Descriptive analyses of entire sample set

ln [pg/ml]	BDG serum
N	1079
Mean	83.39
Median	15.40
SD	233.47
Minimum	0.00
Maximum	2560.74

Table 33: Descriptive statistic of test results of BDG assays Test results in serum samples BDG=Beta-D-Glucan, SD=Standard Deviation

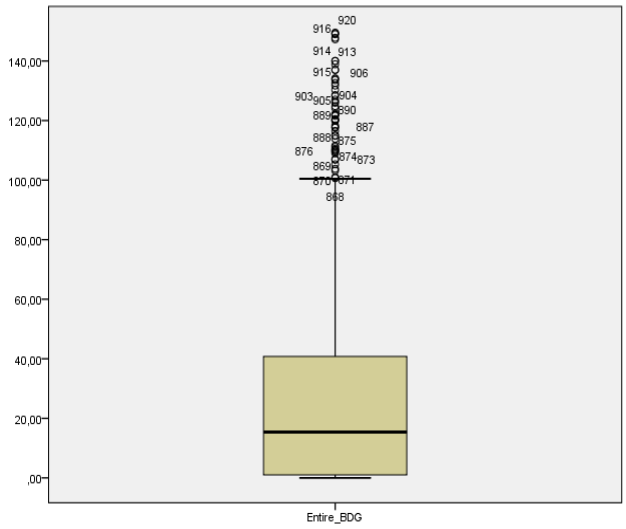

Figure 14: Box plot of BDG assays test results in all serum samples Samples higher than 150pg/ml were deleted for a better overview, BDG=Beta-D-Glucan

6.5.5.2 Descriptive analysis of BDG assay test results in the subgroups

The BDG assay occupies an interim position in fungal differential diagnostic thoughts because it identifies fungal surface markers of *Aspergillus* and *Candida*. For analyses, we used the same subgroups as in the prior analyses for GM assay and *Aspergillus* LFD prototype test. As a reminder, the *Aspergillus* subgroup includes all cases, which matched the *Aspergillus* host factors excluding those with proven invasive candidiasis. Additionally, we excluded all cases with evidence of *Candida* colonization in the upper and/or lower respiratory tract as well as in other body fluids and sites. The *Candida* subgroup includes all cases, which were positive for the *Candida* risk factors or had a proven invasive candidiasis/candidemia excluding the cases with probable IPA. The *Aspergillus* subgroup comprised 924 samples and 125 cases excluding the *Candida* colonization cases. The

Candida subgroup comprised 975 samples and 130 cases after exclusion of the probable IPA cases.

ln [pg/ml]	*Aspergillus* BDG	*Candida* BDG
N	924	975
Mean	98.13	90.84
Median	19.74	20.00
SD	261.21	2560.74
Minimum	0.00	0.00
Maximum	2560.74	2560.74

Table 34: Descriptive statistic of test results of BDG assays in the *Aspergillus* and *Candida* subgroup in serum samples BDG=1,3-β-D-Glucan, SD=Standard Deviation

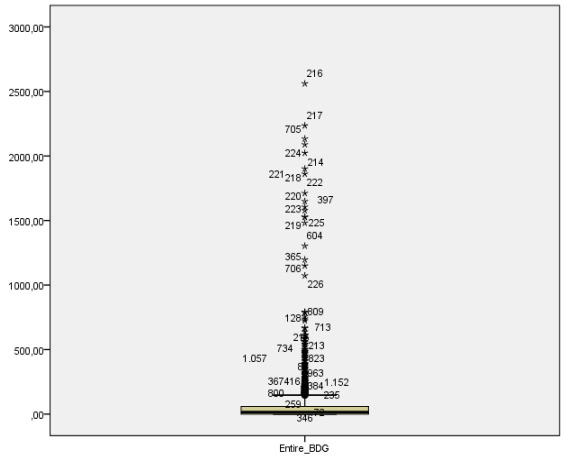

Figure 15: Box plot of BDG assays test results in all serum samples BDG=Beta-D-Glucan

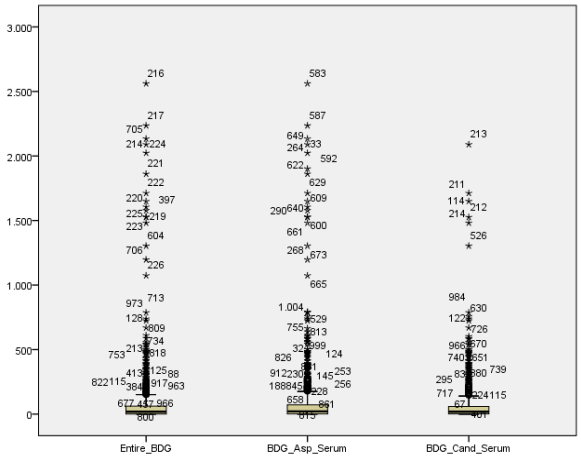

Figure 16: Box plots of the frequencies in *Aspergillus* and *Candida* subgroup BDG=1,3-β-D-Glucan, Asp=Aspergillus, Cand=Candida

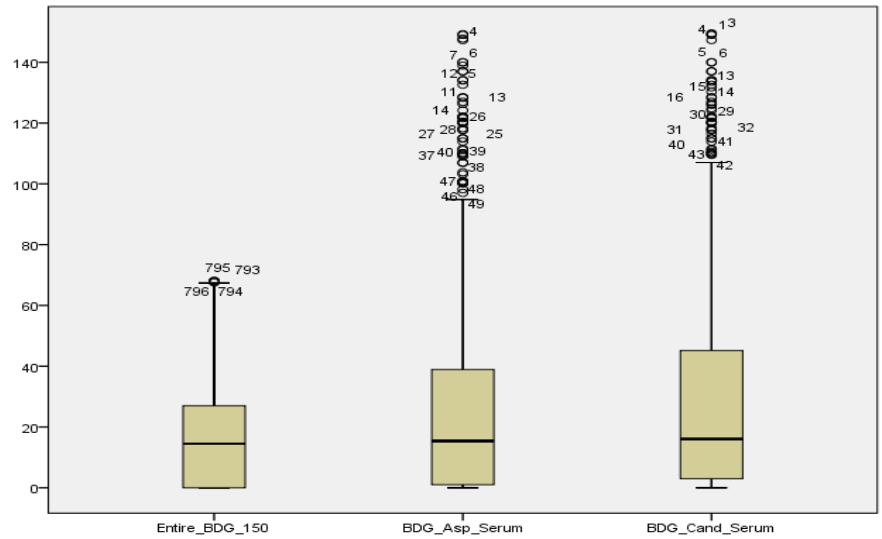

Figure 17: Box plots of the of beta-d-glucan distribution in *Aspergillus* and *Candida* subgroups Samples higher than 150pg/ml were deleted for a better overview BDG=1,3-β-D-Glucan, Asp=Aspergillus, Cand=Candida

The box plots (Figures 17) show the BDG assay test results of the entire serum sample set (box plot 1, Entire_BDG), the assay test results of all serum samples of the *Aspergillus* subgroup (box plot 2, BDG_Asp_Serum), and the whole sample set of the *Candida* subgroup (box plot 3, BDG_Cand_Serum).

We present the BDG assay test results in the *Aspergillus* subgroup, which we additionally classified according to the modified EORTC/MSG guidelines, in Table 35. Furthermore, we present the corresponding box plots in Figure 18 and 19. The first box plot shows the BDG assay test results in cases without any signs and symptoms of an IPA (BDG_No_IPA). The second box plot presents the assay test results of the possible IPA subgroup (BDG_Poss_IPA) and, finally, the third box plot highlights the assay test results of the probable cases (BDG_Prob_IPA).

ln [pg/ml]	No IPA	Possible IPA	Probable IPA
N	657	192	75
Mean	86.28	68.99	124.64
Median	15.4	16.5	8.5
SD	255.41	174.21	310.77
Minimum	0.00	0.00	0.00
Maximum	2560.74	2022.75	2133.30

Table 35: Descriptive statistic of test results of BDG Assays in the *Aspergillus* subgroup BDG=1,3-β-D-Glucan, SD=Standard Deviation, IPA=Invasive Pulmonic Aspergillosis

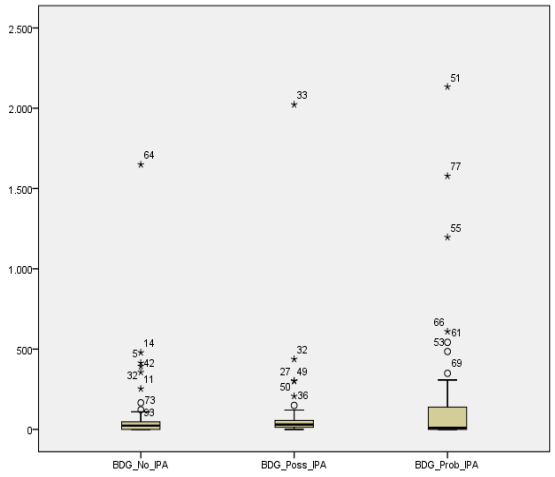

Figure 18: Box plots of the distribution of test results in *Aspergillus* subgroup BDG=1,3-β-D-Glucan, Poss=Possible, Prob=Probable, IPA=Invasive Pulmonic Aspergillosis

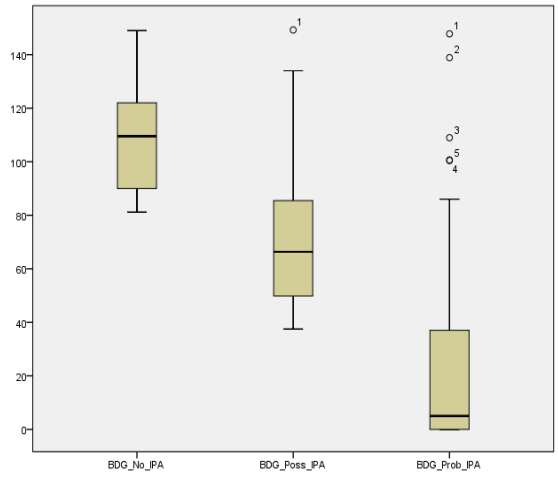

Figure 19: Box plots of the distribution of test results in *Aspergillus* subgroup Samples higher than 150pg/ml were deleted for a better overview BDG=1,3-β-D-Glucan, Poss=Possible, Prob=Probable, IPA=Invasive Pulmonic Aspergillosis

We now focus on the *Candida* subgroup. We showed in table 36 the descriptive evaluation of the BDG assay in three subgroups: Cases without any signs and symptoms of an invasive candidiasis and without *Candida* colonization of the body, cases with proven colonization of the body, and with culture proven candidemia. Figure 20 presents these test results as box plots. The first box plot shows the cases without any signs and symptoms for *Candida* (BDG_No_Cand), the second shows the box plot of cases with colonization (BDG_Colonized_Candida), and the last box plot shows the assay test results of cases with proven candidemia (BDG_Proven_Cand).

In [pg/ml]	No *Candida*	Colonization	Proven Candidemia
N	809	140	26
Mean	57.02	57.12	158.71
Median	15.4	20.94	123.42
SD	133.21	91.61	171.52
Minimum	0.00	0.00	0.00
Maximum	1649.10	587.35	669.35

Table 36: Descriptive statistic of test results of BDG assays in the *Candida* subgroups BDG=1,3-β-D-Glucan, SD=Standard Deviation

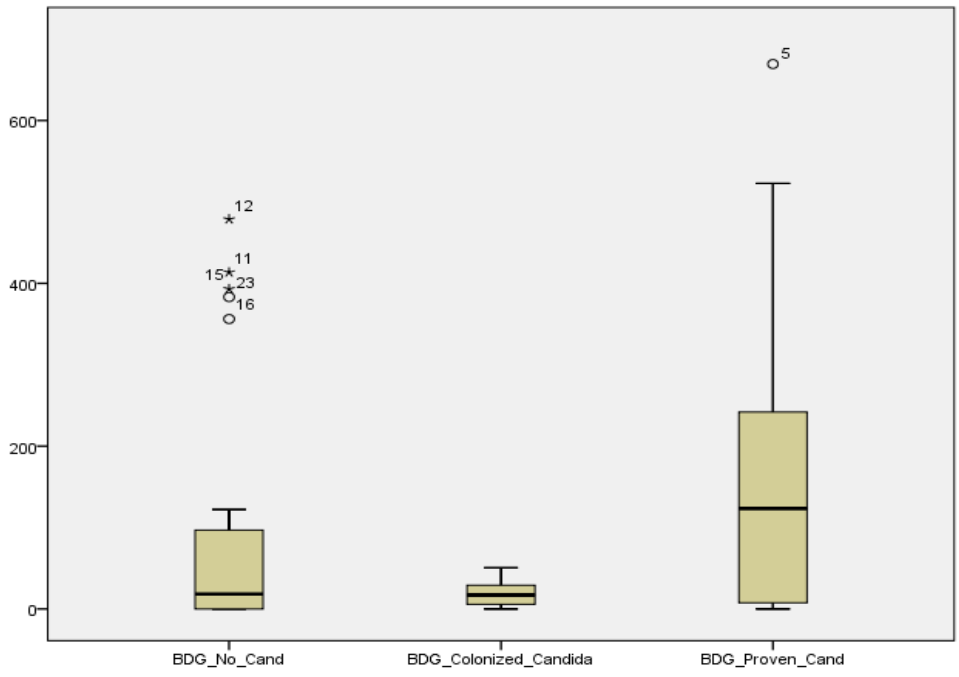

Figure 20: Box plots of the distribution of test results in *Candida* subgroups BDG=1,3-β-D-Glucan, Cand=Candida

6.5.5.3 Performance of BDG Assay Test Results

In 80/125 (64%) of *Aspergillus* cases, the test results were positive, of which 74/80 (92.5%) were classified as false-positive and, 6/80 (7.5%) as true-positive. Possible reasons for this are shown in table 45 and 46. The remaining 45/125 (36%) cases remained negative. Five of these 45 (11.11%) cases were classified as false-negative by comparing the test results to clinical behavior and laboratory work-up, but the reason for false-negativity was not found. Finally, 40/45 (88.89%) cases were true-negative. The established cutoff value to define a sample as positive for BDG was ≥80pg/ml.

Aspergillus cases	FP	TP	FN	TN
Probable IPA	0	6	5	0
Possible IPA	16	0	0	6
No IPA	58	0	0	34

Table 37: Test results of BDG assays in *Aspergillus* subgroup on serum samples FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, IPA=Invasive Pulmonic Aspergillosis

Aspergillus cases	Sensitivity	Specificity	PPV	NPV
Probable IPA	54.5%	N/A	100%%	N/A
Possible IPA	N/A	27.3%	N/A	100%
No IPA	N/A	37.0%	N/A	100%
Summary	54.5%	35.1%	7.5%	88.9%

Table 38: Performance of BDG assay in the *Aspergillus* subgroup on serum samples PPV=Positive Predictive Value, NPV=Negative Predictive Value, IPA=Invasive Pulmonic Aspergillosis, N/A=Not Ascertainable

Aspergillus cases	FP	TP	FN	TN
Possible IPA	7	0	0	9
No IPA	20	0	0	38

Table 39 Test results of BDG assay in *Aspergillus* subgroup for two consecutive serum samples FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, IPA=Invasive Pulmonic Aspergillosis

Aspergillus Cases	Sensitivity	Specificity	PPV	NPV
Possible IPA	N/A	0%	N/A	0%
No IPA	N/A	65.5%	N/A	80.9%
Summary	N/A	63.5%	N/A	83.9%

Table 40: Performance of BDG assay in the *Aspergillus* subgroup of two consecutive possible samples PPV=Positive Predictive Value, NPV=Negative Predictive Value, IPA=Invasive Pulmonic Aspergillosis, N/A=Not Ascertainable

In 81/130 (62.3%) of *Candida* cases, the test results were positive, of which 78/81 (96.29%) were classified as false-positive and 3/81 (3.71%) as true-positive. The remaining 49/130 (37.7%) cases were negative. No case was classified as false-negative and 49/49 (100%) cases were true-negative.

Candida cases	FP	TP	FN	TN
Proven candidemia	0	3	0	0
Colonization	11	0	0	5
No candidiasis	67	0	0	44

Table 41: Test results of BDG assays in *Candida* subgroup on serum samples FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative

Candida cases	Sensitivity	Specificity	PPV	NPV
Proven candidemia	100%	N/A	100%	N/A
Colonization	N/A	31.3%	N/A	100%
No candidiasis	N/A	39.6%	N/A	100%
Summary	100%	38.6%	3.7%	100%

Table 42: Performance of BDG in the *Candida* subgroups on serum samples PPV=Positive Predictive Value, NPV=Negative Predictive Value, N/A=Not Ascertainable

In Tables 43 and 44, we present the performance of BDG assay in serum samples regarding two consecutive positive test results of cases. For this, we only focused on cases, which were classified as false-positive. The cases classified as true-positive also had proven candidiasis and, therefore, are not shown here.

Candida cases	FP	TP	FN	TN
Colonization	6	0	0	5
No candidiasis	23	0	0	46

Table 43: Test results of BDG assays in *Candida* subgroups in serum samples for two consecutive measured positive test results FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative

Candida cases	Sensitivity	Specificity	PPV	NPV
Colonization	N/A	45.5%	N/A	100%
No candidiasis	N/A	66.7%	N/A	100%
Summary	N/A	62.8%	N/A	100%

Table 44: Performance of BDG assays in *Candida* subgroup in serum samples for two consecutive measured positive test results FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, N/A=Not Ascertainable

Possible reasons for false-positive assay test results are summarized in tables 45 and 46. As we know, reasons may be co-reaction with other microorganisms and/or with concurrent applications (anti-thymocyte globulins, procedures such as dialysis).

Clinical features	Number
Blood cultures positive for bacterial microorganisms	23
Acute kidney injury	7
Anti-thymocyte globulines	6
Parenteral nutrition	4
Chronic kidney injury	2
Dialysis	2
Total parenteral nutrition	1

Table 45: Clinical features Clinical features occurred at least one week around the false-positive BDG test results

Microorganisms	Number
Staphylococcus epidermidis	8
Enterococci	4
Staphylococcus hominis	3
Escherichia coli	2
Pseudomonas species	2
Acinetobacter species	1
Stenotrophomonas maltophilia	1
Streptococcus oralis	1
Staphylococcus auricularis	1

Table 46: Microorganisms cultivated in blood cultures simultaneous to false-positive BDG assay test results A total of 21 blood cultures were positive for bacterial microorganisms, some of these were positive for more than one microorganism simultaneously. BDG=1,3-β-D-Glucan

6.5.6 1,3-β-D-glucan assay test results in BAL fluid samples

In this chapter, we describe the test results of the BDG assay in BAL fluid samples. For this, we present the results in the *Aspergillus* subgroup (and according to the modified EORTC/MSG guidelines). We also show the descriptive evaluation of BDG assay in serum samples and present the test results with box plots.

Furthermore, we present the test results in the *Candida* subgroup in the previously defined and used groups (no signs and symptoms of *Candida*, *Candida* colonization, and proven candidemia).

6.5.6.1 Descriptive analyses of entire sample set

In this trial, the BDG assay was performed on 25 BAL fluid samples. Table 47 and Figure 21 present the entire data set.

In [pg/ml]	BDG serum
N	25
Mean	164.85
Median	19.7
SD	257.34
Minimum	0.00
Maximum	930.7

Table 47: Descriptive statistic of test results of BDG assays test results in BAL fluid samples BDG=Beta-D-Glucan, SD=Standard Deviation

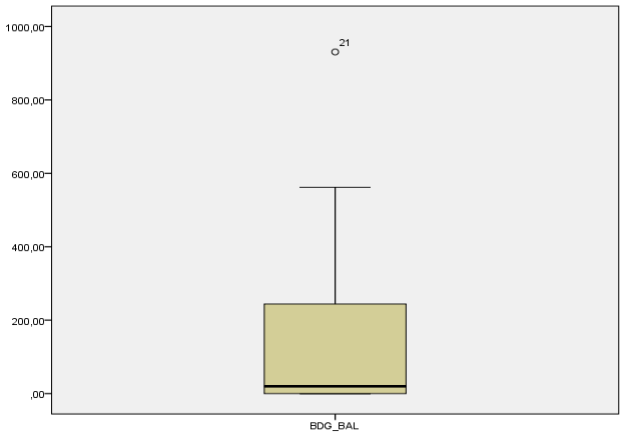

Figure 21: Box plot of BDG assays test results in all BAL fluid BDG=Beta-D-Glucan

6.5.6.2 Descriptive analysis of BDG assay test results in the *Aspergillus* subgroup

In this subgroup-analysis, we excluded all samples with a proof of *Candida* species in cultures made from the BAL fluid samples, thus, we had to exclude 12 samples from further evaluations due to the presence of *Candida* strains.

In [pg/ml]	No IPA	Possible IPA	Probable IPA
N	6	4	3
Mean	103.38	107.52	215.70
Median	20.95	0.00	127.40
SD	217.50	215.05	270.87
Minimum	0.00	0.00	0.00
Maximum	546.60	430.10	519.70

Table 48: Descriptive statistic of test results of BDG assays in the *Aspergillus* subgroups BDG=1,3-β-D-Glucan, SD=Standard Deviation, IPA=Invasive Pulmonic Aspergillosis

The box plots of Figure 22 present the assay test results in BAL fluid samples. The first box plot shows the entire sample set (BDG_BAL_Asp_Subgroup). The second box plot represents the subgroup without signs and symptoms of an IPA (BDG_BAL_No_IPA). The third box plot shows the assay test results in the possible *Aspergillus* subgroup (BDG_BAL_Poss_IPA) and the fourth of the probable subgroup (BDG_BAL_ProbIPA).

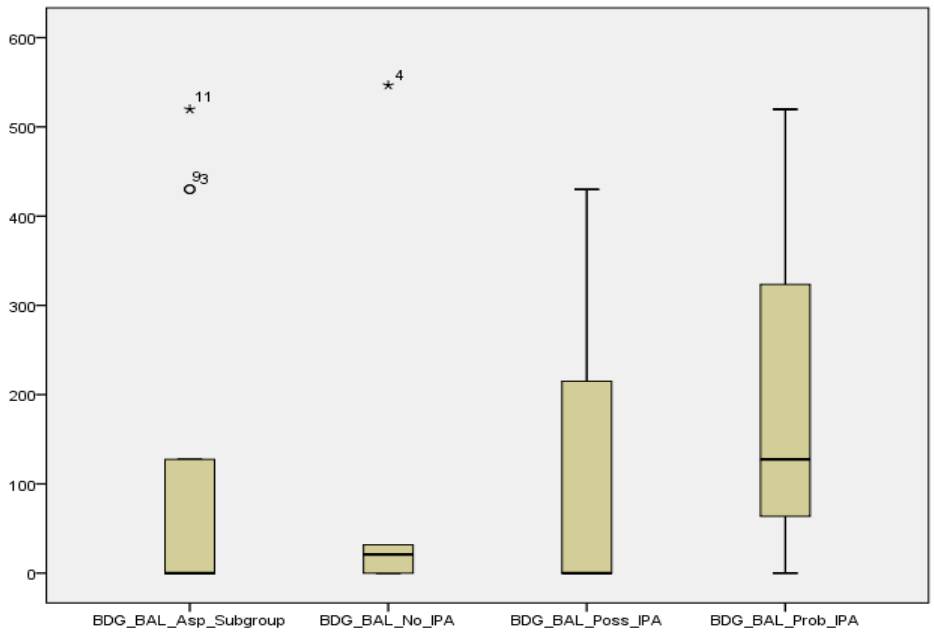

Figure 22: Box plots of BDG assay test results in *Aspergillus* subgroups BDG=1,3-β-D-Glucan, BAL=Bronchoalveolar Lavage, IPA=Invasive Pulmonic Aspergillosis, Poss=Possible, Prob=Probable, IPA=Invasive Pulmonic Aspergillosis

Table 49 shows the BDG assay test results in the concurrent serum samples of the 25 BAL fluid cases.

In [pg/ml]	No IPA	Possible IPA	Probable IPA
N	89	88	27
Mean	46.53	95.12	22.12
Median	11.36	20.74	5.00
SD	99.36	151.45	36.75
Minimum	0.00	0.00	0.00
Maximum	478.70	786.83	162.63

Table 49: Descriptive statistic of test results of BDG assays in the *Aspergillus* subgroups of the concurrent serum samples BDG=1,3-β-D-Glucan, SD=Standard Deviation, IPA=Invasive Pulmonic Aspergillosis

6.5.6.3 Descriptive analysis of BDG assay test results in the *Candida* subgroups

For this subgroup-analysis, we used only BAL fluid samples, which were not classified as probable IPA, so that we had to exclude 5 samples from further evaluations. No patient with a proven candidiasis received bronchoscopy and BAL.

Seven patients had proven *Candida* colonization confirmed by culture from BAL fluid. The remaining 13 cases did not show signs and symptoms of candidiasis.

In [pg/ml]	No *Candida*	Colonization	Proven Candidemia
N	13	7	0
Mean	85.53	253.23	
Median	0.00	36.2	
SD	189.19	363.72	
Minimum	0.00	0.00	
Maximum	564.60	930.60	

Table 50: Descriptive statistic of test results of BDG assays in BAL fluid samples in the *Candida* subgroup BDG=1,3-β-D-Glucan, SD=Standard Deviation

A Figure with box plots could not be calculated for *Candida* cases presented in Table 50 due to a low sample size with invalid values.

6.5.6.4 Performance of BDG assay test results in BAL fluid samples and in Aspergillus and Candida subgroups

The BDG assay test results were evaluated, on the one hand, regarding the classified *Aspergillus* subgroup and in direct comparison to the GM assay test results in serum and in BAL fluid samples. On the other hand, considering *Candida*, we compared culture results, which were made from BAL fluids and positive for *Candida* strains, to the assay results. Assay test results were considered as positive if serum levels were ≥80pg/ml.

Out of 25 BAL samples, we excluded 12 samples from further evaluation due to suspicion for *Candida* contamination. In 4/13 (30.77%) samples, the BDG test results were positive, and 2/4 (50%) of these cases were classified as false-positive due to lack of signs and symptoms of IFI or fungal colonization. One of these BAL fluid samples was, in addition to the BDG assay positive for *Lactobacillus*, *Microbacterium oxydans/maritypicum* and the second sample was positive for *Legionella* PCR. The remaining 2/4 (50%) were classified as true-positive due to probable IPA case or proven *Candida* colonization (proven by culture made from BAL fluids). In 9/13 (69.23%) cases, the test results remained negative and 1/13 (7.69%) sample was classified as false-negative (due to a positive test result for

GM assay in a symptomatic patient). All other cases were classified as true-negative (92.3%).

Aspergillus cases	FP	TP	FN	TN
Probable IPA	0	2	1	0
Possible IPA	1	0	0	3
No IPA	1	0	0	5

Table 51: Test results of BDG assays in *Aspergillus* subgroups in BAL fluid samples IPA=Invasive Pulmonary Aspergillosis, FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, BDG=1,3-β-D-Glucan, BAL=Bronchoalveolar Lavage

Aspergillus cases	Sensitivity	Specificity	PPV	NPV
Probable IPA	66.7%	N/A	100%	N/A
Possible IPA	N/A	75%	N/A	100%
No IPA	N/A	83.3%	N/A	100%
Summary	66.7%	80%	50%	88.9%

Table 52: Performance of BDG in the *Aspergillus* subgroups in BAL fluid samples IPA=Invasive Pulmonary Aspergillosis, PPV=Positive Predictive Value, NPV=Negative Predictive Value, N/A=Not Ascertainable, BDG=1,3-β-D-Glucan, BAL=Bronchoalveolar Lavage

Candida cases	FP	TP	FN	TN
Colonization	0	3	4	0
No Candidiasis	2	0	0	11

Table 53: Test Results of BDG assays in *Candida* subgroups in BAL fluid samples FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, BDG=1,3-β-D-Glucan, BAL=Bronchoalveolar Lavage

Candida cases	Sensitivity	Specificity	PPV	NPV
Colonization	42.9%	N/A	100%	N/A
No Candidiasis	N/A	84.6%	N/A	100%
Summary	42.9%	84.6%	60%	73.3%

Table 54: Performance of BDG in the *Candida* subgroups on BAL fluid samples PPV=Positive Predictive Value, NPV=Negative Predictive Value, N/A=Not Ascertainable, BDG=1,3-β-D-Glucan, BAL=Bronchoalveolar Lavage

Summary	FP	TP	FN	TN
All Cases	2	6	5	12

Table 55: Test results of BDG assays in *Aspergillus* and *Candida* subgroups in all BAL fluid samples FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, BDG=1,3-β-D-Glucan, BAL=Bronchoalveolar Lavage

Summary	Sensitivity	Specificity	PPV	NPV
All Cases	54.5%	85.7%	75%	70.6%

Table 56: Performance of BDG in the *Aspergillus* and *Candida* subgroups PPV=Positive Predictive Value, NPV=Negative Predictive Value, BDG=1,3-β-D-Glucan

6.5.7 Mannan antibody assay in Candida subgroups

Parts of this data were published in *Duettmann Wiebke, Koidl Christoph, Krause Robert, Lackner Gertrude, Woelfler Albert, Hoenigl Martin* **Specificity of mannan antigen and anti-mannan antibody screening in patients with haematological malignancies at risk for fungal infection.** Mycoses. 2016 Jun;59(6):374-8. doi: 10.1111/myc.12482. Epub 2016 Feb. Data, which were partly evaluated in this publication, were also included into the whole data set and used within the thesis with permission of the Journals.

This information is for mannan antibody, mannan antigen, and the combined evaluation of both assays test results.

In total, the assay was performed on 1055 samples, of which 130 cases met the criteria for the *Candida* subgroup of this study.

AU/ml	MAB
Mean	6.89
Median	2.65
SD	12.5
Minimum	0.00
Maximum	80.0

Table 57: Descriptive statistic of test results of mannan antibody assays in *Candida* subgroups SD=Standard Deviation, MAB=Mannan Antibody Assay

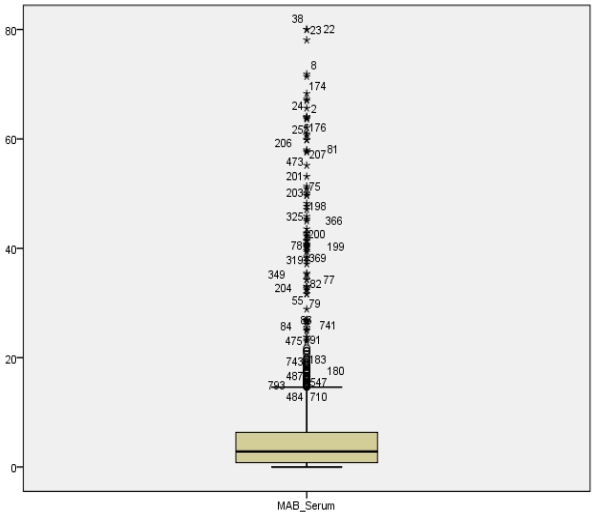

Figure 23: Box plots of mannan antibody assay in *Candida* subgroups

The test results were considered to be positive at levels ≥10AU/ml, so that 181/1055 (17.2%) samples were positive, and the remaining 874/1055 (82.8%) samples remained negative. The evaluation for false/true-positive or false/true-negative samples is only possible in the context of the MAG assay test results due to a general high positive rate of MAB in humans. Consequently, we evaluated the performance of the MAB assay test results only in combination with the MAG assay. However, known reasons for false-positivity are high concentrations of >60g/L of human gamma-globulins, or samples, which are concurrently positive for rheumatoid factor, anti-ds-DNA antibodies, and anti-*Aspergillus* lgG. No participant met these criteria.

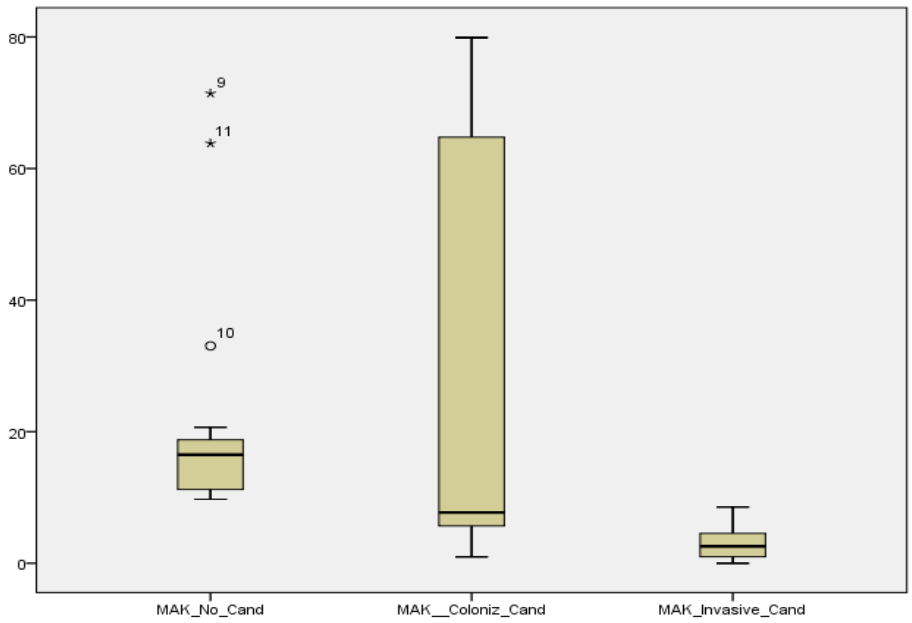

Figure 24: Descriptive statistic of test results of MAB assay in *Candida* subgroups MAB_No_Cand includes participants without any signs and symptoms of Candida, MAB_Coloniz_Cand includes those with Candida colonization, and MAB_Invasive_Cand comprises all Samples of Patients with an invasive Candidemia

6.5.8 Mannan antigen assay in Candida subgroups

Parts of this data were published in *Duettmann Wiebke, Koidl Christoph, Krause Robert, Lackner Gertrude, Woelfler Albert, Hoenigl Martin* **Specificity of mannan antigen and anti-mannan antibody screening in patients with haematological malignancies at risk for fungal infection.** Mycoses. 2016 Jun;59(6):374-8. doi: 10.1111/myc.12482. Epub 2016 Feb. Data, which were partly evaluated in this publication, were also included into the whole data set and used within the thesis with permission of the Journals.

A total of 1138 serum samples were tested for the MAG assay, of which 1050 samples were assigned to the *Candida* group, which included 133 cases. Unfortunately, we could not collect samples of one of the three cases with proven candidemia.

pg/ml	MAG
Mean	21.11
Median	6.43
SD	67.13
Minimum	0.00
Maximum	>500.00

Table 58: Descriptive statistic of test results of MAG assay test results SD=Standard Deviation, MAG=Mannan Antigen Assay

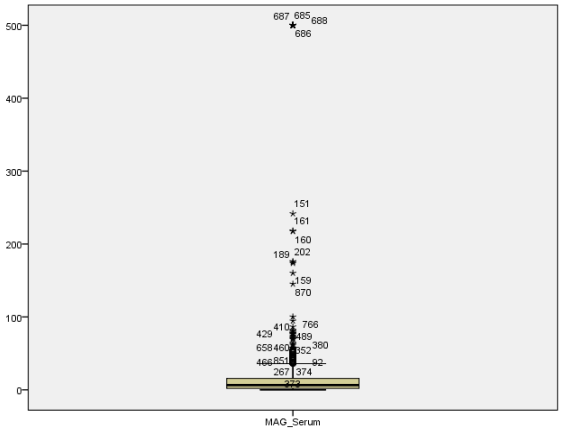

Figure 25: Box plots of mannan antigen assay in *Candida* subgroups

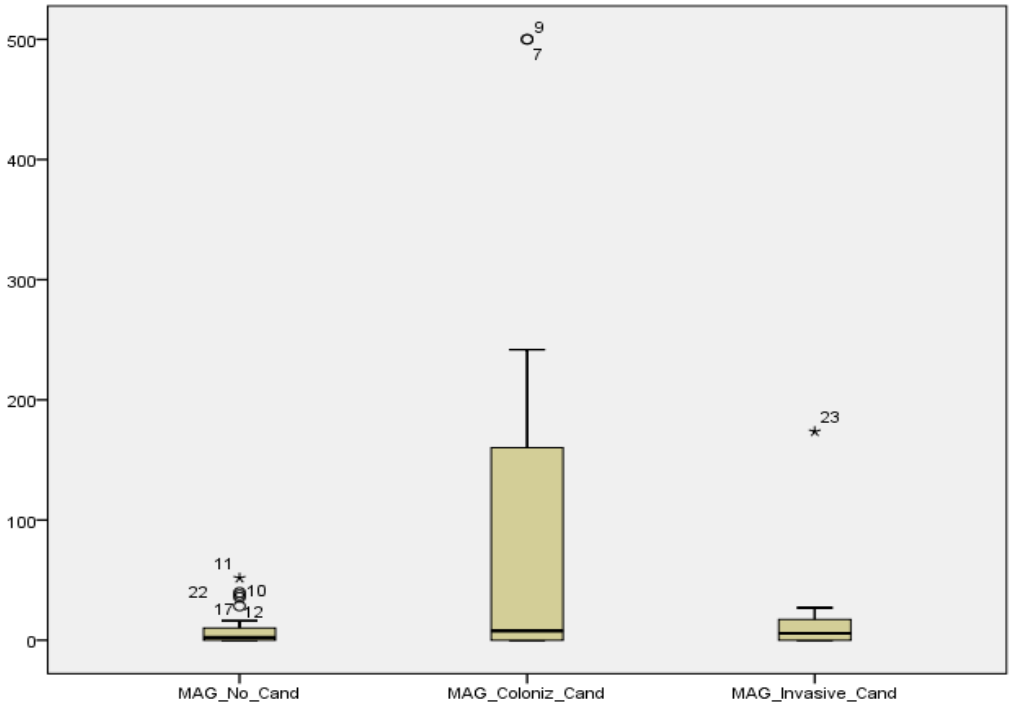

Figure 26: Descriptive statistic of test results of MAG assay in *Candida* subgroups MAG_No_Cand includes participants without any signs and symptoms of Candida, MAG_Coloniz_Cand includes those with Candida colonization, and MAG_Invasive_Cand comprises all Samples of Patients with an invasive Candidemia

In general, samples were considered positive at serum levels ≥125pg/ml, so that 5 (3.76%) cases were found to be positive and from that number 4/5 (80%) cases were categorized as false-positive based on normal mycological laboratory work-up. The known reasons for false positivity are diverse, such as infusions containing hydroxymethyl starch plasma expanders that took place before blood drawing, or human gamma globulins with levels ≥60g/l, which are reactive to anti-toxoplasma antibodies. However, no participants matched these features. One case was categorized as true-positive due to signs and symptoms as well as a positive blood culture. In 128 (96.24%) cases remained negative. In one of these cases, a candidemia, proven by blood culture, was diagnosed but the test

results remained negative. Thus, we classified the corresponding samples as false-negative. Reasons for that could not be found. The remaining 127/128 (99.22%) cases were classified as true-negative.

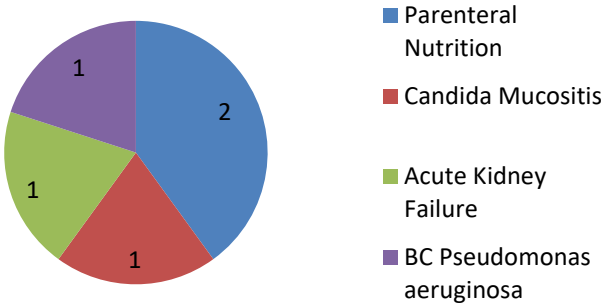

Figure 27: Possible reasons for false positivity in MAG assay test results in *Candida* subgroups BC=Blood Culture, MAG=Mannan Antigen, cases are presented in absolute numbers

Candia cases	FP	TP	FN	TN
Proven candidemia	0	1	1	0
Candida colonization	0	0	0	17
No invasive candidiasis	4	0	0	110

Table 59: Test results of mannan antigen assays FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, BDG=1,3-β-D-Glucan

Candida cases	Sensitivity	Specificity	PPV	NPV
Proven candidemia	50%	N/A	100%	N/A
Candida colonization	N/A	100%	N/A	100%
No invasive candidiasis	N/A	96.5%	N/A	100%
Summary	50%	95.65%	14.29%	99.25%

Table 60: Performance of mannan antigen assays PPV=Positive Predictive Value, NPV=Negative Predictive Value, N/A=Not Ascertainable

6.5.9 Combination of mannan antibody and mannan antibody assays

Parts of this data were published in *Duettmann Wiebke, Koidl Christoph, Krause Robert, Lackner Gertrude, Woelfler Albert, Hoenigl Martin* **Specificity of mannan antigen and anti-mannan antibody screening in patients with haematological malignancies at risk for fungal infection.** Mycoses. 2016 Jun;59(6):374-8. doi: 10.1111/myc.12482. Epub 2016 Feb. Data, which were partly evaluated in this publication, were also included into the whole data set and used within the thesis with permission of the Journals.

The data was analyzed per case and only 4 cases were classified as false-positive (1 with proven colonization and 3 with without no signs of candidiasis) with no recognizable cause (blood cultures taken multiple times and all negative for *Candida*). In the 3 cases of proven candidemia, the combined evaluation of the MAG/MAB assay test results remained negative or no test results were obtained. In detail, for the first patient with

proven candidemia neither the MAB nor the MAG assay could be performed due to admission to the ICU. Regarding the second patient, MAG assay test results were positive, the MAB remained negative and the third patient with proven candidemia showed no positive MAB or MAG assay test results.

Candida cases	FP	TP	FN	TN
Proven candidemia	0	0	2	0
Colonization	1	0	0	16
No invasive candidiasis	3	0	0	111

Table 61: Test results of MAG/MAB assays FP=False-positive, TP=True-positive, FN=False-negative, TN=True-negative, MAG=Mannan Antigen Assay, MAB=Mannan Antibody Assay

Candida cases	Sensitivity	Specificity	PPV	NPV
Proven candidemia	0%	N/A	0%	N/A
Colonization	N/A	94.1%	N/A	100%
No invasive candidiasis	N/A	97.4%	N/A	100%
Summary	0%	96.9%	0%	98.4%

Table 62: Performance of the combined test results of the MAG and MAB assays PPV=Positive Predictive Value, NPV=Negative Predictive Value, MAG=Mannan Antigen Assay, MAB=Mannan Antibody Assay, N/A=Not Ascertainable

5. Discussion

We observed the comparison of the performance of the *Aspergillus* and *Candida* biomarkers in varying tissues. Our focus laid on GM and BDG assays in serum and in urine samples. Additionally, the combined evaluation of MAG assay and MAB assay was confirmed for the early diagnosis of invasive candidiasis in patients suffering from hematological malignancies. The newly developed *Aspergillus* LFD prototype test and the BDG assay were also objected in BAL samples. Finally, a conclusion was drawn about the usefulness of these tests for early diagnosis in clinical day-to day life.

5.1 Performance of GM assay test results in urine samples is comparable to that of the GM assay test results in serum and BAL fluids samples to diagnose IA

The GM assay is a well evaluated biomarker assay in serum and partly well evaluated in BAL fluid samples. Its use in urine samples was a new and much needed approach for long-time monitoring to avoid multiple blood vessel punctures of severely sick patients in order to increase life-quality.

The importance of this study was that, on the one hand, to the best of my knowledge, no other trial investigated the performance of GM assay in urine specimen in adult patients with hematological malignancies. On the other hand, we evaluated the participants of this trial closely, who matched the *Aspergillus* host factors, over a period of eleven months. We performed the GM assay in 202 urine samples and in 1109 serum samples prospectively. We classified our patients strictly according to the modified EORTC/MSG criteria to enable comparison with other studies.
Despite this, was the collection of the urine samples itself comprised with numerous difficulties and led to the weaknesses of this study. Although we obtained many urine samples, we could not collect more due to the dependency on the effort of the participants. To obtain a full sample set from our participants, we also used urine from a urinary catheter. Additionally, the GM assay testing was performed with a delay of one to four days mostly. Old urine und those from Foley catheters could have been gone through an accelerated degradation processes of the galactomannan molecules, which in turn could have led to false-negative assay test results. However, this complicated setting mirrors the reality of daily life in hospitals and gives answers to the question whether the GM assay is useful for routine monitoring in neutropenic patients with hemato-oncological diseases.

The advantage of the use of urine samples for the monitoring of IA lies in the non-invasive collection. Studies showed that the early diagnosis of IA in neutropenic patients was accomplished best specifically on the basis of monitoring, at least twice per week [120, 126]. Currently, serum samples are the basis for this monitoring. Hemato-oncological patients, who are mostly anemic and severely ill, could be spared having to undergo invasive procedures such as multiple blood vessel punctures, Port-a-Catheter system punctures or the implantation of a CVC. This might sound minor, but permanent needle punctures for blood tests decreases life-quality of patients. Furthermore, the necessity of bronchoscopy/BAL might be reduced or from time to time even avoided. If urine proves the validity for diagnosis or better, for the exclusion of disease, in future the tests can aim urine as sample for routine home-POC testing.

On the other hand, the use of urine specimen for GM assay had two main disadvantages. Firstly, the cutoff we chose to consider a urine sample as positive had not been confirmed yet. The kit formulas of the *Aspergillus* Ag Assay by Bio-Rad had specific concentrations and were created primarily for serum and BAL fluid samples. It was not proven that these specific chemicals of the formula, chemical concentrations, and devices could isolate galactomannan in urine samples properly and in a manner that would make it comparable to the other specimens [130]. The second main disadvantage of the use of urine samples for GM assay testing could be the amount of galactomannan in the different body fluids. With respect to an IPA, the highest *Aspergillus* amounts are in the lung (halo sign, air-crescent sign). When the *Aspergillus* conidia starts to grow and infects lung tissue, an IPA occurs. These conidia can be collected by BAL procedure (BAL fluid sample). If the IPA progresses, the *Aspergillus* hypha would penetrate the vessel system of the lung. Only when this step is done would *Aspergillus* enter the blood system and would be detectable in blood system (serum sample) by GM assay. The study by Bennett *et al.* in rabbits and rats described what happens next: After exposure (direct application into vessel system) the galactomannan is enriched in the liver by 30%- to 35% after one hour. Not until 24 hours later was 35% galactomannan measurable in the urine of the rabbit animal model [131]. If the human body processes the galactomannan in the same manner as rats and rabbits, a high amount of galactomannan would be present in the liver around one hour after *Aspergillus* conidia had been broken in the blood system. This fungemia could lead to fever and a septic condition. However, regarding the urinary tract system, only 35% of galactomannan reached the bladder and not until a whole day had passed. However, “early” diagnoses should be available earlier than 24 hours.

In comparison to the urine samples, the advantages of using the GM assay in serum samples were that it had already been well evaluated and it also had been recommended

by the EORTC/MSG committee in their modified guidelines from 2014 for the diagnosis of IA/IPA [34]. Currently, the GM assay is the best diagnostic tool we have in serum (and BAL fluid) samples.

The disadvantages of the GM assay in serum samples were the high variability of GM assay performance. For instance, the sensitivity ranges reportedly from 29%- to 100% as Thornton *et al.* summarized [122]. However, many trials found sensitivities ranging between 40%- to 50%. Multiple reasons for this could not been fully explored, possibly due to the quick initiation of empiric antifungal therapy or prophylaxis [132-134]. In spite of this, Pfeiffer *et al.* stated in 2006 that the GM assay would work well regarding diagnosis and monitoring of IA and can be positive about a week earlier than the medical imaging in patients with hematological malignancies [135]. Nevertheless, the EORTC/MSG guidelines recommend GM assays for the diagnosis on the basis of tests performed twice per week [120]. Hence, the galactomannan kinetics were found as feasible for the early diagnosis and decision making concerning the treatment with antifungal drugs (early change of drug class, intensification, de-escalation of dosage or even discontinuation), if it were monitored regularly over time [136]. Moreover, it takes at least 4- to 6 hours of strict processing in the laboratory to receive GM assay test results [120], which is too long, as the early initiation of antifungals are the key to reduce mortality in IA/IPA. In the daily routine at hospitals, “time to test results” can last much longer (up to 12 hours). Furthermore, to perform the GM assay perfectly special devices (such as cooker and washing machine, which are expensive and need additional space in the laboratory) were necessary and the personnel needed extensive technical skills. And, we only performed assay testing on urine samples until August 23rd 2012. Afterward, we only tested urine samples, if the concurrently taken blood sample had a G assay test result higher than 0.3ODI. Thus, we do not have a full sample set to analyze.

The crucial advantage of GM assay in BAL fluid samples is that it is an established diagnostic tool recommended by the EORTC/MSG guidelines and currently the only reliable method to diagnose an IPA in patients with hemato-oncological diseases. The disadvantages of the GM assay in BAL fluid samples were that it had shown variable performances in prior studies. The sensitivity and specificity ranged from 50%- to 90% (Table 64) within the subgroup of patients with hemato-oncological malignancies. The variation was even higher if other subgroups would also be considered (*e.g.* patients after lung transplantation). Additionally, to collect BAL fluid samples is not as simple because it is an invasive procedure with certain contraindications and complications. Moreover, the *Aspergillus* lesions in the lung must be flushed by sodium solutions, which are

complicated; so that BAL fluid samples tend to have false-negative test results (the affected area within the lung was not flushed thoroughly).

Foremost, the main disadvantage for the evaluation of biomarkers for the diagnosis of IPA itself, which must always be kept in mind, is the lack of a sufficient gold standard to compare with. Plenty of effort and investigations have been undertaken in the search for a safe diagnosis of a proven IA/IPA or at least a satisfying diagnostic pattern [46, 137]. To cultivate *Aspergillus* species from blood cultures is very difficult, on the one hand. Therefore, autopsy-driven trials have been conducted and revealed promising test results of GM assay. Despite this, Maertens *et al.* published as early as in 1999 that the sensitivity and specificity of GM assay test results in serum samples were 92.6% and 95.4%, the PPV and NPV were 93% and 95%. Maertens *et al.* compared the GM assay test results directly to culture and histology from lung tissues of the deceased participant [138]. The performance was promising but not perfect. In mouse models infected directly with *Aspergillus terreus,* only 78% of the mice showed positive GM test results in serum samples. Although, the IPA itself was confirmed by a culture from the mash of the mouse lungs ten days after infection [139].
On the other hand, cultivating *Aspergillus* species from BAL fluid samples is very difficult as well and shows poor results [32]. Recently, Lass-Flörl *et al.* attempted to obtain pure specimens from immunosuppressed patients with newly developed suspicious signs in chest CT scan by using computed tomography-guided percutaneous lung biopsies. The biopsies were processed with Calcofluor white staining and served as gold standard. Compared to the pure biopsy specimen, the GM assay showed sensitivity and a specificity of 88% and 94%, the PPV was 94%, respectively, this includes medical imaging and laboratory work-up, [46], which is satisfying.

Regarding GM assay test results in urine samples, a handful of studies in animal models or in children urine, samples were evaluated with promising test results [140-142]. Presently, no studies have published the performance of GM assay in urine samples of adult patient with hemato-oncological malignancies. In 2014, Duettmann *et al.* searched for the optimal ODI for considering a urine sample as positive. The ODI of 0.1 seemed to be preferable for the diagnosis of disease. It showed the best sensitivity and a specificity of 47.6% and 86%, NPV, PPV and DOR were 94.5% and 24.4%, and 5.57 (95% CI of 2.18-14.22) [121]. We used the same cutoff, as the Youden´s index indicated the most feasible results for the 0.1ODI in a sample-to-sample comparison. We found a sensitivity and specificity of 50% and 61.5% as well as a PPV and NPV of 13% and 91.4%. In a case based evaluation. In a direct sample-to-sample comparison (urine vs. serum and BAL fluid

samples), we found a sensitivity and specificity of 50% and 83.6%. We also found PPV and NPV of 20.51% and 95.18%.

We found a high false positivity rate in the subgroup without any signs and symptoms of an IA/IPA. Reasons for that might be, if patients would receive IV-flushes with fluids containing plasmalyte. At the Department of Hematology at the University Hospital of Graz plasmalyte were generally not used. Hence, no participant was flushed or infused with plasmalyte before blood was drawn for the GM assay testing. Furthermore, antibiotic drugs, which are known to interact with the GM assay test results, were not administered to the participants. Tazobactam/piperacillin was administered 39 times, but no simultaneous positive GM assay test result occurred. On the other hand, we found a high rate of false-negative probable IPA cases (compared to assay test results in BAL fluid samples). For this, the use of antimold-active antifungals on GM assay test results might have an impact [143]. In our study, almost all participants received antifungal drugs. Four patients had samples, which were classified as false-negative: 2/4 of them received posaconazole, 1/4 voriconazole and 1/4 received no antimycotic drug. Due to the low sample set, no sustainable conclusion could be drawn.

Unfortunately, we only had 3 true-positive samples so that the sensitivity and PPV of the GM in urine samples were poor (50% and 13%). The specificity was mediocre helpful (61.5%) and, in comparison, our test results suggested that the NPV was feasible for ruling out IA (91.4%). The reasons for this could be that the manufacturer had not established the GM assay itself for the diagnosis of IA (including IPA) in urine specimen. Additionally, a useful cutoff to consider a urine sample as positive has not yet been found. Furthermore, it is not clear whether enzymes in the blood or urine degrade galactomannan. We found only low levels of GM assay test results in urine specimen that suggest the degradation of the galactomannan molecule occurs before entering or in the urinary tract system. Despite these findings, it is also possible that the galactomannan molecule is not able to pass the Glomeruli of the kidneys. Generally, the galactomannan molecule is fiber-like and cannot be degraded by the GI tract enzymes. It is secreted by *Aspergillus*, has a molecular weight of 18kDa- to 70kDa [144] and is a neutrally charged molecule [145]. Considering the fact that the human kidneys preferably excrete molecules smaller than 10kDa- to 70kDa and molecules with a negative electric charge [146] it seemed unlikely that an aspergillosis would lead to excretion of high amounts of the galactomannan molecules in the urine. Unless the barrier function of the kidney is disturbed due to additional reasons. Moreover, as we mentioned above, in a rat and a rabbit model, only 35% of galactomannan directly applied to the blood system reached the urinary tract system and only after 24 hours. On the other hand, false positivity may occur

in urine samples due to interactions, as described with *Penicillium*, *Alternaria*, *Paecilomyces*, *Geotrichum*, *Mycoplasma pneumonia*, and *Histoplasma* species. Only one participant was colonized with *Geotrichum* and *Alternaria* simultaneously, but the same participant was not positive for fungal biomarkers during his complete stay at the hospital so that the colonization did not seem to influence the GM assay test results. Additionally, it has been proven that galactofuranose-containing foods as well as galactomannan-containing foods such as muesli can lead to higher GM assay test results and thus to false-positivity. However, we did not document this information.

In comparison, the performance of GM assay in serum samples showed sensitivity and specificity of 63.6% and 87.9%, furthermore, the PPV and NPV were 36.8% and 95.6%. In a Cochrane Database Systematic Review by Leeflang *et al*. from 2008, a highly variable average sensitivity of 78% (70%- to 85%) and the specificity of 85% (78%- to 91%) were found to be constantly high [147]. Additionally, White *et al.* described the sensitivity of 85.7%, a specificity of 79.2%, a PPV of 37.5, and a NPV of 97.4% [148]. Held *et al.* found a sensitivity and specificity of 40% and 89%, PPV and NPV of 28.6% and 93.1% as well as a DOR of 3.64 [149]. In all studies, participants were classified strictly according to the modified EORTC/MSG criteria and a cutoff of 0.5ODI was taken to consider samples as positive. Additionally, the study population consisted of patients with hemato-oncological diseases.

We found a high false positivity rate in the subgroup without any signs and symptoms of an IA/IPA. Additionally, we found a high rate of false-negative probable IPA cases (compared to assay test results in BAL fluid samples). Reasons for false-positive or false-negative test results of the GM assay were the same as for urine samples so that we refer to it. However, we want to describe more detailed the possible impact of antimold-active antifungals on GM assay test results [143] because this could have had an impact on the performance of the GM assay. Almost all participants received antifungal drugs. However, in this trial four patients had samples which are classified as false-negative, 2/4 of them received posaconazole, 1/4 voriconazole and 1/4 received no antimycotic drug. In spite of this, the experimental trial by Wiederhold *et al.* showed that neither posaconazole nor voriconazole (as well as caspofungin and LAB) had an influence on GM in serum samples [124]. Due to our low sample set, no sustainable conclusion could be drawn.

Regarding the performance of BAL fluid samples, we had the sensitivity and specificity of 83.3% and 100%, the PPV and NPV were 100% and 94.7% in this study. However, in a meta-analysis by Gou *et al.* from 2010 regarding patients with proven IA/IPA, sensitivity and specificity of 94% and 79%, positive likelihood ratio (PLR) and negative likelihood

ratio (NLR) of 4.41% and 0.07%, were published [150]. In a trial by Hoenigl *et al.* from 2014, the sensitivity and specificity of 82% and 95%, the NPV and PPV of 93% and 88% were found for patients with probable or proven IPA vs no IPA [50]. In comparison, in another 2014 trial by Affolter et al. the sensitivity and specificity were 50% and 73%, PPV and NPV were 16% and 93% [151]. These studies were chosen due to their comparability: they worked with the same high-risk population and they categorized them according to the modified EORTC/MSG criteria. All trials chose the cutoff of 0.5ODI for considering a BAL fluid sample as positive. To explain, for BAL fluid samples a cutoff of 1.0ODI had been recommended by scientists [152], whereas Bio-Rad itself recommended a general cutoff of 0.5ODI [120]. Since most hematological patients were immunosuppressed and since they received antifungal drugs, we preferred the cutoff of 0.5ODI.

One reason for false negativity in the GM assay test result in BAL fluid samples might be the fact that only 300µL were necessary for the assay. If the sample was not blended properly, it may have missed the *Aspergillus* strains. Additionally, since BAL fluids can be very viscous it can be difficult to blend or even to fill the pipette tip properly. There was the option of treating special viscous BAL fluid samples with the reagent Sputasol® (by Thermo SCIENTIFIC) for liquefaction. In 2015, Prattes *et al.* published that Sputasol® reduced the amount of galactomannan in samples and should not be used for diagnostics [153]. Another point is the ongoing discussions about the impact of mold-active antifungal medications, which were administered in 83.33% of our participants. In an experimental trial, Wiederhold *et al.*, proved that antimold-active medications did not have an impact on the GM assay in BAL fluid samples in an animal model. They found a 78.8% interlaboratory agreement for BAL fluid samples [124]. Unfortunately, no participant received fluconazole, which is not anti-mold active, so that we had no samples to compare directly with each other. In addition, the low sample size makes any statement difficult. As we only had one false-negative sample, we did not have any crucial problems with this aspect.

The hypothesis that the GM assay test results in serum and BAL fluid specimen would be comparable to the assay test results in urine samples could not be confirmed by this trial. However, the overall specificity of 61.5%, accompanied with an NPV of 91.4%, seemed to be promising for the exclusion of an invasive infection with *Aspergillus* in the frame of screening in patients with hemato-oncological malignancies.

5.2 The combination of MAG assay and MAB assay testing is useful for the early diagnosis of invasive candidiasis

With respect to invasive candidiasis, the sensitivity of blood culture ranged from 30%- to 50% [1] and needed several days to show useful results, which were poor. Salvage diagnostic tools are needed to close an important diagnostic hole. In this study the performance of MAG/MAB assay test results was evaluated for the early diagnosis or exclusion of invasive candidiases.

The strength of this trial resides in the fact that the participants were well evaluated and, therefore, co-infections with other microorganisms or interactions could be excluded with some certainty. The BDG assay was performed simultaneously in nearly all serum samples so that the assay test results could be compared directly to the test results of MAG/MAB assays. Furthermore, we collected a large sample size (1135 and 1138 samples) to test on.
The weaknesses of this study were that we collected the serum samples prospectively, but we performed the MAG and MAB assay testing retrospectively, but no later than 6- to 12 months after sample collection. Before that, the tubes had been used to perform the GM assay testing. For that, we opened the tubes, but not under a bench flow working station, which could have caused contamination with *Candida* strains. Furthermore, the samples were stored at only -20°C (and not at a lower temperature). Unfortunately, the samples ran through more than one thawing-freezing cycle, which could have degraded the mannan molecules. One of the most important weaknesses of this study is that only three of our participants suffered from a proven invasive candidiasis. Due to the low sample size, we evaluated in proven candidemia a profound conclusion cannot be drawn [154].

The advantage of the combined analyses of the MAG/MAB assay test results was that it was recommended by the current ESCMID guidelines [110] for the diagnosis of invasive candidiases. It is important to know that it is claimed that the assay test results cannot detect superficial *Candida* infections such as of the skin or mucosa (thrush). If the combined test results of MAG/MAB assays were positive, a deep-seated candidiasis or candidemia could have taken place and the initiation of antifungal therapy was advised as well as long-term follow-ups such as ultrasound of the liver or TTEs. Additionally, to differentiate superficial from deep-seated candidiases or candidemia is also crucial for the choice of the antifungal regime and thus an advantage of the combined evaluation of the assays test results.

The disadvantage of the MAG/MAB assay testing was that a strict processing at a laboratory of at least six hours was necessary to obtain the test results. The laboratory procedures required special devices (such as cooker and washing machine), and extensive technical ability were necessary for carrying out these assay testing. For example, at our laboratory, only two of the medical technical assistants (MTA) were allowed to perform the MAG/MAB assay testing because the assay test results of the other MTA were not credible. The early diagnosis in patients with hematological malignancies was performed specifically based on continuous monitoring, which means two times per week. Scientists and Bio-Rad recommended this regular performance of the assays. Thus, anemic and severely ill patients had to undergo multiple blood vessel punctures, Port-a-Cath system punctures, or the implantation of a CVC was necessary. This comprised extra effort and expense. The more often the MAG/MAB assay test results were needed, the more expensive the *Candida* monitoring became. An MTA was occupied two days per week for the whole working day and the assay kits were expensive.

With regards to the combined evaluation of MAG assay and MAB assay test results, at the third ECIL conference held in 2009 a sensitivity of 83% (mean) and a specificity of 86% (mean) in patients with hematological malignancies was proposed [155]. The MAG/MAB assays were recommended for the detection of candidemia, deep-seated candidiasis, and chronic disseminated candidiasis. With respect to candidemia only, the sensitivity and the specificity of 80% and 85% were described, which revealed a test accuracy of 50%- to 70%. The combined evaluation of the MAG/MAB assays was reported to produce test results, which were positive six days earlier than blood cultures. Additionally, a NPV of >85% was stated [110].

In our study, the performance of the both assay test results were a sensitivity and specificity of 0% and 96.9% as well as an PPV and NPV of 0% and 98.4%. Reason for the poor sensitivity and PPV was with certainty because we had no true-positive sample, which was accounted by the low case rate of proven candidiases and, in order to that, the low sample set. Additionally, the test results of the MAB assay in immunocompromised patients, especially after myeloablative chemotherapies or in HSCT recipients, was difficult because the ability to produce immune globulins decreased or even stopped. This led to a distorted picture of immunoglobulin-induction after contact with *Candida* strains and, in turn, caused false-negative MAB assay test results. Regarding the MAG assay, negative test results do not rule out an infection due to possible low concentrations and the rapid elimination of mannan antigens during infections.

Despite this, the specificity of 96.9%% and the NPV of 98.4% were promising for ruling

out diseases, as we found in our study. Duettmann et al found same results in a study. [156]. Reasons for false positivity regarding test results of the MAB assay often occur, if samples contained >60g/L of human gamma-globulins or would be positive for rheumatoid factor, anti-ds-DNA antibodies or anti-*Aspergillus* Ig G. Since we did not evaluate our serum samples to these factors, we only cannot exclude them as cause. On the other hand, the MAG assay could produce false-positive test results in samples which also contain human gamma-globulins (≥60g/L) or which would be reactive to anti-toxoplasma antibodies and cross-reactions due to simultaneous infusions with hydroxymethyl starch plasma expanders have been reported. We also did not evaluate our serum samples for these factors and cannot draw any conclusions on the reasons of false positivity.

With regards to the MAG assay only, the third ECIL conference stated a sensitivity of 58% (mean) and a specificity of 93% (mean) for the Platelia™ *Candida* Ag Plus in patients with hemato-oncological malignancies. No dependency to neutropenic phases has been proposed [155]. In our IFI study, we found sensitivity and a specificity of 50% and 95.65%, a PPV and a NPV of 14.29% and 99.25% for the MAG assay test results. Mikulska *et al.* showed, that the assay test results were heterogenic depending on the causative species. The sensitivity was highest for *Candida albicans* followed by *Candida glabrata*, and *Candida tropicalis*. Furthermore, the MAG assay test results seemed to be positive for about six days (mean) earlier than blood cultures in 78% of patients with proven candidemia. Additionally, the MAG assay test results were positive approximately 18 days (mean) earlier in patients with hepatosplenic invasive/deep-seated candidiasis [155]. Interestingly, data suggested that the MAG assay would be very sensitive and even detected superficial candidiasis such as thrush [157], what our data could not confirm.

The presence of mannan antibodies in the blood system is not easy to judge because the *Candida* mannan is a ubiquitous molecule, which is widespread in the air and on surfaces. Hence, making the decision of whether the MAB assay test result was true-positive or true-negative was not possible, thus we could not perform a clarification and performance evaluation. Additionally, patients with high-dose chemotherapies or even stronger myeloablative therapies could lose the ability to produce antibodies. A study by Mikulska *et al.* showed that the MAB assay test results were heterogenic, as it was also in our study, and had varying ranges depending on the *Candida* species. The highest sensitivities have been found for *Candida albicans* followed by *Candida glabrata* and *Candida tropicalis* [155].

This trial admittedly had a large sample size but only three cases of proven candidemia. The performance of the combined evaluation of the MAB assay and the MAG assay test

results does not seem feasible. The MAG assay alone showed better target values than the combined evaluation. Thus, we suggest that it would be possible to save costs due to patient monitoring by deploying the MAG only in order to reduce antifungal agents in a prophylactic manner.

5.3 Performance of *Aspergillus* LFD prototype and BDG assay in BAL fluids samples for the early diagnosis of IPA or to rule out pulmonary IFI

We evaluated the *Aspergillus* LFD prototype test and the BDG assay in this trial in BAL fluid samples with the aim to determine its usefulness for the safe and early diagnosis of IPA or IFI compared to the already established GM assay in BAL fluid and serum samples. Here the two hypotheses "*Performance of the Aspergillus LFD prototype test results is comparable to that of the GM assay test results in BAL fluids and serum to diagnose IPAs*" and "*BDG assay levels in BAL fluid samples are useful for the early diagnosis of IFI and/or to rule out pulmonary IFI*" should be addressed.

The strength of this study observing BDG assay in BAL fluid samples was the thorough evaluation, which helped to reduce the impact of interfering glucans, co-infections with bacteria or other fungi, and contaminations caused otherwise [158]. With regard to the *Aspergillus* prototype test, one of the main strengths of this trial was the fact that all participants were classified strictly according to the revised EORTC/MSG criteria and that a cutoff of 0.5ODI was chosen to consider a BAL fluid sample (as well as a serum sample) as positive due to the patients with immunosuppression.
The weaknesses of this trial were the fact that multiple freeze and thaw cycles (room temperature and -70°C) were necessary to receive all test results. Apart from the fact that the collection of BAL fluid samples is in itself not a sterile procedure, the opening of the sample tube - especially if it is not done under a flow bench working station as well as a multiple drive in with non-sterile pipette tips - to take some of the specimen for the *Aspergillus* LFD prototype test. The BDG assay test, and the GM assay test, might lead to further contamination via airborne microorganisms. Generally, contamination with interfering glucans played an important role in interpreting the BDG assay test results, because many products used at hospitals and laboratories contain 1,3-beta-D-glucans. For example, when we started this IFI trial, the BDG assay showed extremely high-test results. In time, contamination of the reagent Grade Water was discovered, and the bottles were exchanged. We retested the affected samples and then had more credible test results. Moreover, the Fungitell instruction manual warns about protein degradation

due to long freezing cycles. Rose *et al.* confirmed in 2014 that the 1,3-beta-D-glucan showed a poor reproducibility in BAL fluid samples over time even if stored in freezers at once [159], which was a fact in our study procedures. Finally, in this trial, we performed the BDG assay and the *Aspergillus* LFD prototype in only 25 samples, which might have resulted in an alpha bias [154]. Additionally, one more weakness was that we used two different sample tubes for the BDG assay testing and the *Aspergillus* LFD prototype test (and the GM assay testing). This could have led to varying test results and made sample-to-sample interpretations difficult. Reasons were possible contaminations (different treatment of two different tubes) and that the sample might have come from differently flushed areas of the lung.

The advantages of the BDG assay itself resided in its pan-fungal approach [122]. The 1,3-beta-D-glucan is an important protein for fungal cell surfaces. Almost every fungus including *Aspergillus*, *Candida*, *Pneumocystis*, *Fusarium*, and *Scedosporium* produces it. Exceptions are *Mucorales*, *Cryptococcus*, and *Blastomyces* dermatitidis. However, *Cryptococcus neoformans* and *Blastomyces* dermatitidis produce only low levels of 1,3-beta-D-glucan and, therefore, they are mostly not detected by the BDG assay [126]. Hence, a positive BAL assay test result in a sample can raise suspicion for an IFI of the lung caused by the above-mentioned microorganisms. In some cases, it is the only laboratory tool to detect a contamination or infection compared to plate cultures made from BAL fluid samples. The test procedure itself takes around an hour [126] and the confirmed test result can be transmitted to the attending medical doctor quickly. Furthermore, the use of BAL fluid specimens, compared to blood samples, could reduce the impact of systemic factors, which lead to false-positive assay test results, such as the simultaneous application of drugs [124] and ongoing bacteremia [160-162].
The advantages of the *Aspergillus* LFD test prototype were that it was easy to run and did not require extra equipment. Test results were obtainable only after a short period (available within 15 minutes). As far as is known, the *Aspergillus* LFD prototype test did not cross-react with drugs or other contaminants, thus, did not cause false-positive test results as was the case for other biomarkers such as the GM- and the BDG assays [163]. Moreover, the *Aspergillus* LFD prototype test detects the antigen jk5 on the surface of the active growing *Aspergillus* conidia, which makes the important differentiation between colonization and infection possible [122]. This made this LFD prototype test more useful than *Aspergillus* PCR and the GM assay. Furthermore, it has been stated that the antifungal prophylaxis or therapies with antimold-active substances (such as voriconazole, posaconazole, caspofungin or LAB), whose impact on GM assay test results is highly discussed among experts, do not influence the *Aspergillus* LFD prototype test results

[124]. In the meanwhile, the *Aspergillus* LFD prototype test had been available on the market and had been advertised for its proven efficacy to diagnose IPA in BAL and serum samples. Moreover, it seemed to be significantly reproducible compared to BDG and GM assay test results [124].

The BDG assay itself had multiple disadvantages. The procedure of the assay was laborious and required well-educated personnel with special training. All glassware had to be dry heated and depyrogenated at 235°C for at least seven hours prior to use. Extra material (the test tubes for the standard series preparation had to be made of borosilicate glass and the storage tubes had to be glucan-free) and special devices (special incubating plate reader) had to be purchased. That means that the effort before the BDG assay can be offered to doctors at hospitals would be enormous. Furthermore, extra pipette tips without cotton plugs had to be used [126]. Samples, which developed protein clots during a storage phase could not be tested and had to be thrown away. Thus, sample consumption may be high, which can slow down diagnostics and increase effort (new sample had to be obtained from the patient) as well as costs (new sample had to be tested). Additionally, the assay is extremely cost intensive (about 700€ for 96 wells, 16 stripes). With respect to the interpretation of the test result, the BDG assay could not differentiate between fungal species and, thus, cannot lead to a calculated antifungal therapy (different first-line therapies). It cannot make statements about resistances or susceptibilities [122, 126]. Furthermore, the BDG assay is already known for its high false-positivity rate.

An important disadvantage of the *Aspergillus* LFD prototype was that we used a test kit set for research only. Retrospectively, it turned out that there were production problems with some of the charges, resulting in inconsistent kits. Unfortunately, our test kit set was among them.

More disadvantages of both tests were to collect BAL fluid samples is not simple because it is an invasive procedure with certain contraindications and complications, what makes the tests inaccessible for many patients. Moreover, the lesion in the lung must be flushed by sodium solution, which is complicated, so that BAL fluid samples tend to have false-negative test results. Bronchoscopy with BAL is an invasive technique, which requires stable cardiorespiratory patients with stable coagulation. Finally, we want to mention that we used a test kit charge might not be working compared to other charges as we found out after accomplishing the study.

In our IFI study, for the BDG assay test results in BAL fluid samples and regarding the *Aspergillus* subgroup, we found a sensitivity and specificity of 66.7% and 80% as well as a

PPV and NPV of 50% and 88.9%. In comparison, regarding IPA Hoenigl *et al.* published, in 2014, the sensitivity and specificity of 80% and 76%, the PPV, NPV, and DOR were 44%, 94%, and 12.4 (2.3 - 68) [50]. Moreover, Theel *et al.* evaluated, in 2013, 109 participants and found the PPV of 20% and NPV of 83% with regard to IPA and PCP [164]. Regarding the *Candida* subgroup, we found a sensitivity and specificity of 42.9% and 84.6% as well as a PPV and NPV of 60% and 73.3%. Regarding the *both* subgroups, we found a sensitivity and specificity of 54.5% and 85.7%, the PPV and NPV of 75.0% and 70.68%.

The question of the appropriate cutoff in BAL fluids samples and in varying subpopulations such as in hemato-oncological malignancies has not been answered yet. For example, in 2015 Mutschlechner *et al.* published a paper in which the BAL fluid samples were considered to be positive at a cutoff of ≥100pg/ml in recipients of a solid organ transplantation [165]. However, we used the cutoff of 80pg/ml for considering a sample as positive.

In order to classify samples as false-positive or as true-positive, the assay test results of the 25 BAL fluid samples were compared to the other laboratory findings. Reasons for falsely classified samples were presented in the section's weaknesses of the study and disadvantages of the BDG assay itself. To summarize, false positivity can be caused by colonization with fungi on the skin or in the respiratory tract and can be caused by cross-reactivity due to other microorganisms or interfering glucans. Additionally, contaminated or glucan-containing flushing solution or swabs can cause it. On the other hand, false negativity can be caused by dilution effects of the BAL procedure, or if the infected area is missed by the flushes. During the study, we found some reasons for false positivity, but not all, so that we still had to consider the high rate of false-positive test results. However, the BDG assay test results of two cases were classified as false-positive because the laboratory work-up remained normal in healthy participants regarding IA/IPA and invasive candidiasis. The additionally performed plate cultures from the BAL fluid samples were positive for *Lactobacillus*, *Microbacterium oxydans/maritypicum, Enterobacter cloacae, Streptococcus* of the Viridans group, *Enterococcus faecialis* and *Prevotella melaninogenica*. This could suggest co-interactions of the BDG assay. For the interpretation of the BDG assay test results, the following had to be considered: With regard to *Aspergillus* species, the BDG assay was obtained in combination with the GM assay (in serum and in BAL fluid samples), *Aspergillus* PCR, and the Aspergillus LFD prototype test. The *Aspergillus* host factors and the health condition of the patient were crucial. BDG assay test results cannot differentiate between *Aspergillus* colonization or ongoing infection, although even a colonization of the lungs in immunocompromised hosts

with signs and symptoms of an acute pneumonia would lead to an antifungal therapy sooner or later. To interpret the BDG assay test results in BAL fluid samples regarding *Candida*, it is important to know that *Candida* pneumonia occurs mostly secondary to disseminated infections. They are rare because *Candida* is not very pneumotropic [10]. Hence, the presence of *Candida* in the lung would be most likely linked to contamination and to initiate an antifungal therapy here would not be adequate.

On the other hand, in our study, the *Aspergillus* LFD prototype test had a sensitivity and specificity of 0% and 87.5%, PPV and NPV of 0% and of 70%. Unfortunately, as mentioned above, we used an invalid kit set that led to inconsistent test results. Since we performed the IFI trial, this fact came up into light and the test kits were called back. In the meanwhile, the *Aspergillus* LFD test was brought to market as a CE product and shows satisfying test results.

It is not easy to answer the question whether the performance of the *Aspergillus* LFD prototype test was as useful as the GM assay performance in BAL fluids and serum samples. Taking into consideration that the *Aspergillus* LFD prototype test has many practical advantages and that other trials found more promising test results the LFD prototype test would be a significant improvement in the early and quick diagnosis of IPA. Unfortunately, this trial could not confirm prior findings, which was first and foremost due to the charge of the prototype, which has been proven to not work sufficiently after I completed my laboratory work and my thesis.
On the one hand, a pan-fungal biomarker such as the BDG assay with a specificity of 85.7% could be the first, and sometimes the only, laboratory tool to rule out an IFI and, thus, reducing the amount of unnecessary side effects, costs, and the induction of new resistance patterns in fungi. However, a differentiation between *Aspergillus*, *Pneumocystis*, and *Candida* is necessary for the correct initiation of antifungal therapy. Therefore, there is a call for another salvage laboratory tools or laboratory bundles including the BDG assay, GM assay, the *Aspergillus* PCR and the *Aspergillus* LFD prototype test would be a start.

5.4 Conclusion and prospects

In this dissertation thesis, we presented four hypotheses and evaluated five biomarker assay kits in serum, BAL fluid or urine samples. Furthermore, we build up a biobank (samples and information from patients).

The GM assay in serum and in BAL fluid samples proved its solid test results and we can confirm its eligibility for the diagnosis of IPA/IA in patients with hemato-oncological malignancies. Considering the subgroup analysis, our findings showed that in patients with signs and symptoms of an acute pneumonia and/or septic health condition, the performance of the GM assay in BAL fluid samples showed perfect test results by differentiation of the cases as possible or probable. Additionally, the GM assay test results in BAL fluid samples and in the concurrent serum sample set of one case were approximately for 0.75ODI higher than in cases classified as possible IPA or no IPA. For comparison, the most false-positive test results were found in the subgroup of “No IPA”, which means in patients without any signs and symptoms of an acute onset of severe infectious disease. In summary, the GM assay test results should undermine clinical findings and help with deciding whether an antifungal therapy would be necessary or not.

The GM assay performed in urine samples seemed to be useful in ruling out fungal diseases by having specificity 61.5% and NPV of 91.4%. Again, we used an unconfirmed cutoff of 0.1ODI to evaluate the urine samples as positive compared to the classification of the cases according to the revised EORTC/MSG criteria by serum and BAL fluid samples. This conclusion should be considered with caution. The implementation could ease the screening for IPA in already severely ill patients and help to maintain life-quality by avoiding permanent needle punctures.

The combined evaluation of MAB assay test results and MAG test results seems not to be feasible for diagnosis of deep-seated or invasive candidiasis, but the MAG assay alone or in a combination with MAG assay and BDG assay with regard to *Candida* infections might be interesting and will hopefully be evaluated in another study.

Our data showed that the *Aspergillus* LFD prototype test, although it has much practical potential, did not fulfil our expectations. Reason was probably that we used a charge for first testing and ours had been broken, as we found out later. The now available *Aspergillus* LFD test is a validated product and finds its use in clinical day-life.

The BGD assay in BAL fluid samples seemed to be very sensitive about fungal microbes, although contamination rate is generally high in BAL fluids, which had an impact on the performance. Thus, the usefulness in early diagnosis seemed to be low, especially as the BDG assay cannot differentiate between the varying species. In the meanwhile, more studies confirmed these findings [166]. However, we propose a higher cutoff (90 or 100pg/ml) - in serum and in BAL fluid samples - to reduce the false-positivity rate.

Since the biomarkers should be conducted twice per week – except the *Aspergillus* LFD test - the idea is to replace the prophylactic initiation of antifungal drugs in neutropenic patients with high risk profiles for empiric and pre-emptive schedules in order to reduce costs, side-effects, and fungal-resistances. The pre-emptive schedule has already been attempted in clinical trials, which showed promising results [167]. We can support this idea since the patients with signs and symptoms of an infection (acute pneumonia or sepsis) and signs in CT scans showed the best assay performances.

6. Limitations

In this chapter, we give an overview of potent limitations in the biomarker assays.

6.1. PlateliaTM *Aspergillus* Ag kit by Bio-Rad

1. Mold-active antimycotic drugs may influence the assay results and decrease the sensitivity.
2. The drug interacts with other classes of fungi such as *Penicillium*, *Alternaria*, *Paecilomyces*, *Geotrichum*, and *Histoplasma*. However, with the routine laboratory work-up, we tried to reduce this bias.
3. False positivity can occur and must be considered under the following circumstances:
    a. Galactofuranose, which is an ingredient of cereals, cereal products, cream dessert, wheat, muesli, human milk, and cow milk, can lead to false-positive values. Moreover, patients with disturbed intestinal barrier can develop a galactofuranose antigenemia.
    b. Patients receiving batches of piperacillin/tazobactam products, less frequently of amoxicillin (especially when combined with clavulanic acid and parenteral formulations) products can lead to false-positive results. To recapitulate, the consumption of semi-synthetic β-lactam products should be considered when the test result is positive.
    c. If products that contain GM due to fermentation processes by fungi are taken orally or parenterally (altered intestinal barrier), this intake can lead to higher test results.
4. Plasmalyte^{TM} can lead to false-positive test results and should be avoided for the collection of BAL fluid samples [120].

6.2 Fungitell® assay by associates of CAPE COD Inc.

1. Some individuals show elevated 1,3-β-D-glucan levels which are not explainable with clinical evaluation. This might be due to non-human pathogenic fungi. In this case, a retest is recommended.
2. Positive test results have been found in patients treated with fractionated blood products such as albumin and immunoglobulins.
   Alcohol-soaked gauze used to prepare the site before blood sampling can lead to contamination [126]

6.3 PlateliaTM *Candida* Ab plus by Bio-Rad

1. Negative test results cannot exclude an invasive candidemia, especially in patients with an altered immune system due to an impaired ability to build up antibodies. Further diagnostic tests as well as clinical features must be considered.
2. The test result should be combined with the MAG.
   False-positive test results have been reported in samples which contain >60 g/L of human gamma-globulins and which are positive for rheumatoid factor, anti-ds-DNA antibodies, or anti-Aspergillus IgG [128]

6.4 PlateliaTM *Candida* Ag plus by Bio-Rad

1. Negative test results cannot exclude an infection due to possible low concentration and the rapid elimination of mannan antigen during infection. For a correct interpretation of the test results, further diagnostic tests as well as clinical signs and symptoms must be considered.
2. Negative test results must be interpreted in combination with MAB.
3. Monitoring of patients at high risk with MAG is recommended. Trials showed a higher sensitivity when tested more often.
4. Cross-reactions in patients who received infusions with hydroxymethyl starch plasma expanders have been reported.
   False-positive results can occur in samples containing human gamma-globulins with levels ≥60 g/L and in those which are reactive to anti-toxoplasma antibodies [129]

6.5 *Aspergillus* LFD prototype test by OLM Diagnostics

Cross-reactions with *Emericella amstelodami* and *Emericella nidulans*, *Eurotium*, and *Neosartorya fischeri* have been described. Cross-reactivity with *Penicillium* species (such as *Biverticillium* or *Talaromyces*) and weak cross-reactions with antigens by *Paecilomyces variotii* have been found.

In undiluted serum samples, a negative test result can occur due to the hook effect (the high antigen concentration impairs the antigen–antibody binding). If in a patient an invasive aspergillosis is suspected, who has a negative test result, it should be repeated with a 10-fold diluted sample [122]

6.6 Study design

6.6.1 Invasive aspergillosis

The lack of an appropriate gold standard to compare the biomarker assay test results for the diagnosis of IPA is the main problem. As long as this problem has not addressed, every trial will be built merely on suspicion, and not on facts. This is criticized in detail in the discussion part of this dissertation thesis, but shall be repeated here due to its crucial importance.

The revised EORTC/MSG criteria are constructed for research approach only, but without the revised EORTC/MSG criteria, no structured disease classification is possible at all. To optimize this scientific approach to a clinical sub-setting, each deceased patient should have received an autopsy to confirm the involvement of fungi as a cause of death.

6.6.2 Invasive candidiasis

The diagnosis of invasive candidiasis entails the same gold standard problem. The clinical behavior cannot be distinguished from infections caused by other microorganisms or the underlying hematological diseases. CT scans are not useful and show signs only when *Candida* has settled into an organ. These signs are unspecific and remain speculative until an organ biopsy proves the source of infection. Weighing the advantages and disadvantages of an organ biopsy in patients who might have bleeding disorders would take us too far afield. However, the blood culture is positive in approximately 50% [1] of cases only depending on the infecting *Candida* strain [32].

Further studies to find a working gold standard is warranted.

6.7 Power of the study

The calculation of the sample size by a statistician should be done before a trial starts. Too small sample sizes comprise the danger of overseeing elements of true significance. This can lead to an alpha bias. And too large sample sizes can lead to significant correlations that are not true and, therefore, lead to a beta bias [154]. Importantly this was not a clinical trial but an observational explorative cohort study and therefore no sample or study population size calculations were performed before the start of the study.

7. List of figures

8. List of tables

9. Appendix

Comparison of our assay test results to published results

	(1) Leeflang et al.	(2) White et al.	(3) Held et al.	(4) IFI study
Sensitivity	78%	85.7%	40%	63.6%
Specificity	85%	79.2%	89%	87.9%
PPV	N/A	37.5%	28.6%	36.8%
NPV	N/A	97.4%	93.1%	95.6%
DOR	N/A	N/A	3.64	N/A

Table 63: Comparison of performances of galactomannan assays (Platelia) in serum samples PPV=Positive Predictive Value, NPV=Negative Predictive Value, DOR=Diagnostic Odds Ratio; (1) Leeflang et al. from 2015 [147] (2) White et al. from 2013 [148] (3) Held et al. from 2013 [149] (4) Test results of this trial: IFI

Comparison in IFI study	GM serum	GM BAL fluid	GM urine
Sensitivity	63.6%	83.3%	50.0%
Specificity	87.9%	100%	61.5%
PPV	36.8%	100%	13%
NPV	95.6%	94.7%	91.4%

Table 64: Comparison of galactomannan assay (Platelia) performances GM=Galactomannan Assay in [ODI], BAL=Bronchoalveolar Lavage, PPV=Positive Predictive Value, NPV=Negative Predictive Value

	ECIL MAG	ECIL MAG/MAB	IFI study: MAG	IFI study: MAG/MAB
Sensitivity	58%	83%	50%	0%
Specificity	93%	86%	95.65%	96.9%
PPV	N/A	N/A	14.29%	0%
NPV	N/A	N/A	99.25%	98.4%

Table 65: Performances of MAG assay test results and the combined evaluation of MAG assay and MAB assay test results The cutoff of ≥125pg/ml was chosen for considering the MAG Assay Test Results as positive. For evaluation of the MAB Assay a cutoff of ≥10AU/ml was chosen. All participants were immunosuppressed. ECIL=European Conference on Infections in Leukemia, MAG=Mannan Antigen Assay, MAG/MAB=Combined Evaluation of MAG Assay and MAB (mannan antibody) assay test results, IFI=Invasive Fungal Infection, PPV=Positive Predictive Value, NPV=Negative Predictive Value [168]

	Sensitivity	Specificity	PPV	NPV
Aspergillus	66.7%	80%	50%	88.9%
Candida	42.9%	84.6%	60%	73.3%
Summary	54.5%	85.7%	75.0%	70.6%

Table 66: Performance of BDG assay test results BAL fluid samples in the *Candida* and *Aspergillus* subgroup of IFI study. PPV=Positive Predictive Value, NPV=Negative Predictive Value, IFI=Invasive Fungal Infections

	(1) Hoenigl et al.	(2) Theel et al.	(3) IFI study
Sensitivity	80%	N/A	54.5%
Specificity	76%	N/A	85.7%
PPV	94%	20%	75.0%
NPV	44%	83%	70.6%
DOR	12.4 (2.3-68)	N/A	N/A

Table 67: Comparison of BDG assay (Fungitell®) performances in BAL fluid samples The table represents the performance found by (1) Hoenigl et al. with respect to IPA [50] (2) Theel et al. with respect to IPA and PCP [164] (3) IFI regarding IPA and Candida colonization or candidemia. The cutoff of 80pg/ml was chosen to consider samples positive. All participants were immunosuppressed. IFI=Invasive Fungal Infection, PPV=Positive Predictive Value, NPV=Negative Predictive Value, DOR=Diagnostic Odds Ratio

	(1) Hoenigl *et al.* 2012	(2) Hoenigl *et al.* 2014	(3) IFI study 2012
Sensitivity	100%	80%	0%
Specificity	81%	95%	87.5%
PPV	71%	80%	0%
NPV	100%	95%	70%

Table 68: Performance of *Aspergillus* LFD prototype test in BAL fluid The table represents the target values of other trials, which have the following basic prerequisites: Patients were classified according to the revised EORTC/MSG recommendation, a cutoff of 0.5 ODI had been chosen to consider the BAL fluid samples positive, the participants had underlying hematological diseases; IFI=Invasive Fungal Infection, PPV=Positive Predictive Value, NPV=Negative Predictive Value (1) Hoenigl et al. 2012 [169]; (2) Hoenigl et al. 2014 [50]; (3) IFI study

	(1) Hoenigl *et al.*	(2) Guo *et al.*	(3) Affolter *et al.*	(4) IFI study
Sensitivity	80%	90%*	50%	83.3%
Specificity	98%	94%*	73%	100%
PPV	89%		16%	100%
NPV	95%		93%	94.7%
DOR	160 (12.9-1,984)	N/A	N/A	N/A
PLR	N/A	4.41%**	N/A	N/A
NLR	N/A	0.07%**	N/A	N/A

N/A The table represents the Performance found in trials, which had classified regarding the revised EORTC/MSG Recommendation and a Cutoff of 0.5ODI was chosen to consider the BAL fluid samples positive in participants with hemato-oncological diseases; IFI=Invasive Fungal Infection, PPV=Positive Predictive Value, NPV=Negative Predictive Value, DOR=Diagnostic Odds Ratio; PLR=Positive Likelihood ratio, NLR=Negative Likelihood Ratio; N/A=Not Available (1) Hoenigl et al. 2014 [50] (2) Guo et al from 2010 [150], *regarding patients with probable/proven invasive aspergillosis; **regarding patients with proven invasive aspergollis (3) Affolter et al. from 2014 [151] (4) Test results of this trial

10. Collected data of the participants

1. Surname
2. First name
3. Date of birth
4. Informed consent, yes/no
5. Inclusion study, date
6. Discharge from study
7. Admission day
8. Discharge hospital
9. Sex
10. Department
11. Underlying disease
12. Reason for admission
13. Chemotherapy, drug-name, duration, dosage, application
14. Cortisone, drug-name, duration, dosage, application
15. Prednisone equivalent dosage /day /kg, duration, dosage
16. Body weight
17. T-Zell suppressants, drug-name, duration, dosage, application
18. Immunosuppressive therapy, drug-name, duration, dosage, application
19. Neutropenia, duration, frequency
20. Chest CT scan, interpretation
21. Chest X-ray, interpretation
22. Fever >14 Days, duration, frequency
23. Fever despite broad-spectrum antibiotics, duration, frequency
24. Bone marrow suppression, duration, frequency
25. HSCT
26. GVHD, when present: grade, organ, therapy
27. Autopsy, date, results
28. Antifungal drugs, drug-name, duration, dosage, application
29. Antibiotic drugs, drug-name, duration
30. Posaconazole prophylaxis, duration, dosage
31. Breakthrough infection, date and reason

32. Survival after 4 weeks
33. Survival after 12 weeks
34. Therapy breakthrough infection, drug-name, dosage, duration, response
35. Change of dosage, reason
36. Risk factor
37. Proton pump inhibitor, drug-name, duration, dosage, application
38. Structured one-site patients' education, date, frequency
39. Voriconazole phylaxis/therapy, duration, dosage
40. Change of dosage, reason
41. Risk factor
42. Samples, date, tissue, which test, result
43. Sheets sample collection
44. Day of decease
45. BAL fluid, date, how much 0.9% NaCl solution is used
46. BAL fluid cytology results
47. BAL fluid culture results
48. Colonization with fungi, which body site, microorganisms, date
49. Mucositis, duration, frequency, therapy
50. CVC, duration of insertion, frequency
51. Port-a-cath system, duration of insertion, frequency
52. Parenteral nutrition, duration, frequency
53. Total parenteral nutrition, duration, frequency
54. ICU, duration, frequency
55. Urinary catheter, duration, frequency
56. Febrile neutropenia, duration, frequency
57. Blood culture, duration, frequency, microorganisms
58. Red cell concentration, frequency
59. Platelet concentration, frequency
60. Surgical intervention, reason, date, procedure
61. Dialysis, date, duration, frequency
62. Acute kidney injury, duration, frequency
63. Chronic kidney injury, frequency

64. Diabetes mellitus 2

65. Start and end of invasive ventilation, date

66. Sepsis

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