Dissertation

DIAGNOSTIC POTENTIAL OF IMMUNOLOGIC BIOMARKERS IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES AT RISK FOR INVASIVE MOLD INFECTIONS

Submitted by

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Linz; September 26, 2025

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The consent of Prof. Dieter Buchheidt to use his data and contributions to our co-authored publications in this dissertation could not be obtained as he passed away in 2021. All other principal and co-investigators gave written consent to the usage of their data in this thesis.

In addition, I have obtained the necessary permissions in accordance with good scientific practice standards and copyright regulations if content from other works has been reproduced.

Previous Publications Related to This Thesis

Parts of this thesis have been published in

1) Heldt S, Hoenigl M. Lateral Flow Assays for the Diagnosis of Invasive Aspergillosis: Current Status. Curr Fungal Infect Rep. 2017;11(2):45-51.
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2) Heldt S, Eigl S, Prattes J, Flick H, Rabensteiner J, Pruller F, Niedrist T, Neumeister P, Wolfler A, Strohmaier H, Krause R, Hoenigl M. Levels of interleukin (IL)-6 and IL-8 are elevated in serum and bronchoalveolar lavage fluid of haematological patients with invasive pulmonary aspergillosis. Mycoses. 2017;60(12):818-25.
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3) Heldt S, Prattes J, Eigl S, Spiess B, Flick H, Rabensteiner J, Johnson G, Pruller F, Wolfler A, Niedrist T, Boch T, Neumeister P, Strohmaier H, Krause R, Buchheidt D, Hoenigl M. Diagnosis of invasive aspergillosis in hematological malignancy patients: Performance of cytokines, Asp LFD, and Aspergillus PCR in same day blood and bronchoalveolar lavage samples.
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4) Rawlings SA, Heldt S, Prattes J, Eigl S, Jenks JD, Flick H, Rabensteiner J, Pruller F, Wolfler A, Neumeister P, Strohmaier H, Krause R, Hoenigl M. Using Interleukin 6 and 8 in Blood and Bronchoalveolar Lavage Fluid to Predict Survival in Hematological Malignancy Patients With Suspected Pulmonary Mold Infection. Front Immunol. 2019;10:1798.

Original data have also been presented in part at

1) IDWeek 2016, New Orleans (LA), United States (poster presentation)
2) 11. Österreichischer Infektionskongress (ÖIK) 2017, Saalfelden, Austria (oral presentation)
3) 27th European Congress of Clinical Microbiology & Infectious Diseases (ECCMID) 2017, Vienna, Austria (poster presentation)
4) 51st Annual Meeting of the Deutschsprachige Mykologische Gesellschaft (DMyKG) 2017, Münster, Germany (oral presentation)
5) 8th Trends in Medical Mycology (TIMM) Conference 2017, Belgrade, Serbia (poster presentation)
6) 23rd Congress of the European Hematology Association (EHA) 2018, Stockholm, Sweden (poster presentation)
7) 20th Congress of the International Society for Human and Animal Mycology (ISHAM) 2018, Amsterdam, The Netherlands (poster presentation)
8) Doctoral Day 2025, Graz, Austria (oral presentation).

Usage of Artificial Intelligence Tools

Applications that use artificial intelligence were used solely to improve the language, style, conciseness, and grammar of the text.

The following application was used as a dictionary and for assistance with translation as needed:

DeepL Übersetzer (Starter Pack). DeepL SE: https://www.deepl.com/de/translator.

The following application was used as needed during correction of the draft for suggestions on rephrasing to achieve more concise language, for suggestions on stylistic adaptation of text passages, or for checking for grammatical errors:

DeepL Write. DeepL SE: https://www.deepl.com/de/write

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Contents

Abbreviations and Definitions

A

AA = aplastic anemia

ABPA = allergic bronchopulmonary aspergillosis

aaCD4+ = antigen-activated cluster of differentiation 4 positive

AF = antifungal

AGM-LFA = IMMY sona *Aspergillus* Galactomannan Lateral Flow Assay; a product from IMMY, Norman (OK), United States

AIDS = acquired immunodeficiency syndrome

ALL = acute lymphoblastic leukemia

alloHCT = allogenic hematopoietic cell transplantation

AML = acute myeloid leukemia

ARDS = acute respiratory distress syndrome

AspLFD = *Aspergillus* Lateral-Flow Device; a product from OLM Diagnostics, Newcastle upon Tyne, United Kingdom (formerly; currently IMMY MDX Ltd., Braintree, United Kingdom)

altMacro = alternatively activated macrophage

AUC = area under the curve

B

BAL = bronchoalveolar lavage

BALF = bronchoalveolar lavage fluid

BDG = 1,3-β-D-glucan

BRSC = bronchoscopy

BW = body weight

C

C5 = component 5 of the complement system

CAP = community-acquired pneumonia

CAR = chimeric antigen receptor

CCL = cysteine-cysteine motif chemokine ligand

CCR = cysteine-cysteine motif chemokine receptor

CD = cluster of differentiation

CFU = colony-forming units

CI = confidence interval

classMacro = classically activated macrophage

CLL = chronic lymphocytic leukemia

CMV = cytomegalovirus

CRP = C-reactive protein

CT = computed tomography

CTL = cytotoxic T lymphocyte

CXCL = cysteine-X-cysteine motif chemokine ligand

CXCR = cysteine-X-cysteine motif chemokine receptor

D

DC = dendritic cell

DNA = deoxyribonucleic acid

DOR = diagnostic odds ratio

E

EBV = Epstein-Barr virus

EDP = electronic data processing

EDTA = ethylenediaminetetraacetic acid

EIA = enzyme-linked immunoassay

ELISA = enzyme-linked immunosorbent assay

Endo = endothelial cell

EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group

EORTC-IDG = European Organization for Research and Treatment of Cancer/Infectious Diseases Group

Eos = eosinophile

F

FDA = US Food and Drug Administration

FISH = fluorescence in situ hybridization

FPCRI = Fungal PCR Initiative

G

G-CSF = granulocyte colony-stimulating factor

GM = galactomannan

GM-CSF = granulocyte-macrophage colony-stimulating factor

GVHD = graft-versus-host disease

H

HCT = hematopoietic cell transplantation

HIV = human immunodeficiency virus

HL = Hodgkin lymphoma

HSV = herpes simplex virus type

I

IA = invasive aspergillosis

ICC = intraclass correlation coefficient

IFI = invasive fungal infection

Ig = immunoglobulin

IL = interleukin

ILC = innate lymphoid cell

IL-2R = interleukin 2 receptor

IMI = invasive mold infection

IFN = interferon

IPA = invasive pulmonary aspergillosis

IQR = interquartile range

J

JAK = Janus kinase

K

KAGes = Steiermärkische Krankenanstaltengesellschaft m.b.H. (unofficial translation: Styrian Hospital Association)

L

LB = lower bound

LFA = lateral flow assay

LFD = lateral flow device

LR = likelihood ratio

LR+ = positive likelihood ratio

LR– = negative likelihood ratio

LT = lymphotoxin

M

mab = monoclonal antibody

Macro = macrophage

MAIT = mucosal-associated invariant T cell

Mast = mast cell

Max = maximum

MDS = myelodysplastic syndromes

mEpi = mucosal epithelial cell

Min = minimum

MM = multiple myeloma

MNC = mononuclear cell

MRI = magnetic resonance imaging

MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group

MSGERC = Mycoses Study Group Education and Research Consortium

MWU = Mann-Whitney *U*-test

N

nCD4+ = naïve cluster of differentiation 4 positive

Neutro = neutrophile

NFL = negative feedback loop

NHL = non-Hodgkin lymphoma

NKC = natural killer cell

NKTC = natural killer T cell

NPV = negative predictive value

O

ODI = optical density index

OR = odds ratio

P

PCR = polymerase chain reaction

PCT = procalcitonin

PFL = positive feedback loop

PMF = primary myelofibrosis

PPV = positive predictive value

PRR = pattern recognition receptor

Q

Q1 = 1st quartile/25th percentile

Q3 = 3rd quartile/75th percentile

R

ROC = receiver operating characteristic

RSV = respiratory syncytial virus

S

sIL-2R = soluble interleukin 2 receptor

sIL-6R = soluble interleukin 6 receptor

Sens = sensitivity

Spec = specificity

STAT3 = signal transducer and activator of transcription 3

T

Tfh = T follicular helper cell

TGF = transforming growth factor

Th = T helper

Th* = T helper cell type *

TKI = tyrosine kinase inhibitor

TNF = tumor necrosis factor

TOR = target of rapamycin

Treg = regulatory T cell

U

u vs. pp = unlikely invasive mold infections versus probable and proven invasive mold infections

u vs. ppp = unlikely invasive mold infections versus possible, probable, and proven invasive mold infections

UB = upper bound

up vs. pp = unlikely and possible invasive mold infections versus probable and proven invasive mold infections

Y

YI = Youden's index

List of Figures

List of Tables

Kurzfassung

Hintergrund: Patienten mit hämatologischen Malignomen haben ein erhöhtes Risiko für schwerwiegende invasive Schimmelpilzinfektionen (IMI). Die Diagnostik ist jedoch nach wie vor schwierig.

Ziele: Primäres Ziel waren Bewertung und Vergleich der Testeigenschaften von Interleukin (IL) 4, 6, 8, 10, 15, 17A, 22, löslichem IL-2-Rezeptor, Tumornekrosefaktor α, Interferon γ, Cysteine-Cysteine Motif Chemokine Ligand 5 (CCL5), einer *Aspergillus* Polymerasekettenreaktion (PCR) und dem *Aspergillus* Lateral-Flow Device (AspLFD) zur Diagnose pulmonaler IMIs unter antimykotischer Therapie.

Methoden: In dieser prospektiven beobachtenden Kohortenstudie wurden Blut und bronchoalveoläre Lavage-Flüssigkeit (BALF) von Patienten mit hämatologischen Malignomen und Verdacht auf pulmonale Infektionen untersucht. Die Fälle wurden anhand der Definitionen der European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group von 2008 klassifiziert. Die genannten Biomarker und Assays wurden danach bewertet, ob sie wahrscheinliche/nachgewiesene IMIs von unwahrscheinlichen/möglichen IMIs unterscheiden können.

Ergebnisse: Wir rekrutierten 106 Patienten, darunter 18 wahrscheinliche und eine nachgewiesene IMI. IL-6 und IL-8 in der BALF konnten die Post-Test-Wahrscheinlichkeit einer IMI verringern, IL-8 im Serum erhöhen. IL-17A-Spiegel wiesen in der BALF von IMIs eine andere Wahrscheinlichkeitsverteilung auf, aber keinen beweisbaren diagnostischen Wert. *Aspergillus* PCR und AspLFD mit BALF konnten die Post-Test-Wahrscheinlichkeit einer IMI erhöhen, mit Vollblut oder Serum waren beide Tests aber nicht hilfreich. CCL5-Spiegel im Serum waren auffällig niedrig, jedoch ohne klare Abhängigkeit von IMIs.

Schlussfolgerung: Beim Nachweis von invasiven Aspergillosen waren PCR und LFD überlegen. IL-6 und IL-8 könnten aber bei der Diagnostik von IMIs helfen, die kein Galaktomannan oder β-D-Glucan freisetzen.

Abstract

Background: Patients with hematological malignancies have an increased risk of invasive mold infections (IMI). Despite progress in the past, IMIs are still associated with high mortality and difficult to diagnose.

Aims: Primary objective was to evaluate and compare the test performances of interleukins (IL) 4, 6, 8, 10, 15, 17A, 22, soluble IL-2 receptor, tumor necrosis factor α, interferon γ, cysteine-cysteine motif chemokine ligand 5 (CCL5), an *Aspergillus* polymerase chain reaction (PCR), and the *Aspergillus* Lateral-Flow Device (AspLFD) to diagnose pulmonary IMIs in populations with high prevalence of mold-active antifungal medication.

Methods: In this prospective observational cohort study, blood and bronchoalveolar lavage fluid (BALF) samples from patients with underlying hematological malignancies and suspected pulmonary infections were investigated. Cases were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. Above biomarkers and assays were evaluated to distinguish unlikely/possible and probable/proven IMIs.

Results: We recruited 106 cases, including 18 probable and one proven IMI. IL-6 and IL-8 in BALF were able to decrease post-test probability for IMIs and IL-8 in serum to increase post-test probability. IL-17A levels in BALF of IMIs had different probability distributions but diagnostic potential could not be proven. *Aspergillus* PCR and AspLFD with BALF had potential to increase post-test probability of IMIs but no diagnostic value with whole blood or serum. CCL5 levels in serum were notably low without evidence of an association with IMIs.

Conclusion: PCR and LFD were superior for diagnosing invasive aspergillosis, but IL-6 and IL-8 may help in diagnosis of IMIs not releasing galactomannan or β-D-glucan.

Introduction

Patients with hematological malignancies are at high risk of serious life-threatening infections (1). In immunocompromised patients with pneumonia and underlying hematological diseases, the most identifiable pathogens in bronchoalveolar lavage fluid (BALF) are bacteria, followed by fungi as the second most (2). Formerly, *Candida* infections were the most common invasive fungal infections (IFI) in patients with hematological malignancies, until *Candida*-preventing antifungal prophylaxis became popular (3-5). Since then, invasive aspergillosis (IA) has replaced invasive candidiasis as the most common IFI in these patients (3-5).

Epidemiology and Pathogenesis

Molds are opportunistic pathogens found ubiquitously in the environment (6-8). Most invasive mold infections (IMI) in patients with hematological malignancies are caused by *Aspergillus*, with *Aspergillus fumigatus* being the most frequent causative species for IAs (3, 7, 9-11). Other *Aspergillus spp.* that are known to cause invasive diseases are *A. flavus*, *A. niger*, *A. terreus, A. nidulans*, and *A. lentulus* (7, 10, 12). Besides *Aspergillus*, the next most frequent mold genera belong to the order Mucorales: The most common genus of Mucorales is *Rhizopus* (viz., constituting 50% of mucormycoses); after that, *Lichtheimia* (formerly *Mycocladus* or *Absidia*) and *Mucor* are other frequently occurring genera of Mucorales (1, 13-16). Diseases caused by fungi of the order Mucorales are referred to as mucormycosis. This term has replaced the term zygomycosis, which also included other mold genera that were medically less important (16, 17). Mucorales are followed in frequency by *Fusarium*, *Scedosporium*, and *Lomentospora*, and incidences of *Cunninghamella* have also increased (10, 11, 18). Although much less common, other genera for which occasionally invasive infections in the lower respiratory tract of severely immunocompromised patients were published are *Acremonium*, *Alternaria*, *Exophiala*, *Paecilomyces*, and *Scopulariopsis* (19, 20). However, the local epidemiology regarding prevailing mold genera can be significantly different from place to place (21).

Mortality

The infection-related mortality of febrile neutropenic patients has been reduced to less than 10% through improved empiric therapies and prophylaxes (1). However, mortality varies greatly between the different infectious agents, with mold infections responsible for a high

proportion of infection-related mortality especially in patients that have received a hematopoietic cell transplantation (HCT) (20, 22). The mortality of patients with IA is between 14% and 77% in general, despite adequate antifungal therapy (7, 10, 23). This wide range is explained by various cofactors: For example, IA in allogeneic HCTs is associated with higher mortality rates, while IA in autologous HCTs is associated with comparatively low rates (10, 23). Another cofactor is the time until the diagnosis of IA is made and a therapy is initiated (24). The longer the therapy is postponed (e.g., due to diagnostics with low reliability or long turnaround time), the worse is the outcome (23-25). If an appropriate empiric or targeted treatment is not initiated because an IA is not diagnosed or not considered within the differential diagnoses, the mortality is 100% (7). This applies to other mold genera as well. The mortality of invasive mucormycosis is 20%–87% despite appropriate therapy, and delayed diagnosis is one of several factors for worse prognosis (16, 26-28). Similarly, initiating necessary measures in time is also important for the prognosis of invasive fusariosis which mortality is 33%–100%, particularly depending on the persistence of neutropenia and the continuation of glucocorticoid therapy (5, 29, 30). In addition, invasive lomentosporiasis and invasive scedosporiosis also have high mortalities of 50%–100% and 50%–62%, respectively, despite adequate therapy (29, 31, 32).

Major Mechanisms of Innate Immunity Defending Molds

Molds can infect patients in several ways. Breakdowns or penetrations of the skin barrier are possible routes of infection, but the most common way is through the respiratory tract by inhaling spores (33-35). After inhalation of spores, the incubation period for, for example, IA is between 2–90 days (7, 36, 37). Therefore, IMIs can occur despite appropriate preventive measures in the hospital, as spores may have already been present in the respiratory tract when immunocompromising interventions like chemotherapies or HCTs are started (7). However, the minimum dose of inhaled spores needed to cause an infection is not known (8).

The airway epithelia, phagocytes, and soluble pattern recognition receptors (PRR) (e.g., pentraxins, collectins, and ficolins) play a key role in the defense against mold spores (33, 38, 39). Most inhaled spores are transported out of the lungs via mucus and the cilia of the respiratory epithelium (33, 40, 41). If spores encounter epithelia, they will activate the immune response via PRRs and upregulate production of cytokines and antimicrobial peptides (42). However, *Aspergillus fumigatus* produces mycotoxins, proteases, and

secondary metabolites that slow down ciliary activity, damage the respiratory epithelium, and inhibit phagocytosis (43). Additional underlying conditions like diabetes mellitus, chemotherapy, radiation, or viral infections can also cause epithelial damage, increasing the risk for fungal colonization or invasion of the airways (44-46). Moreover, exposed extracellular matrix proteins can serve as a binding site for spores and support tissue invasion (44, 47).

Type II pneumocytes produce collectins (e.g., surfactant protein-A and surfactant protein-D) that opsonize spores, promoting phagocytosis by alveolar macrophages (9, 38, 48). Alveolar macrophages form the first stage of the cellular immune system for defending molds (9, 49, 50). Apart from collectins, phagocytosis is supported by pentraxins (e.g., pentraxin 3) and ficolins (38, 51-54). However, glucocorticoids hamper phagocytosis, especially at doses that excess 40 mg of prednisone per day (or other glucocorticoids equivalently dosed) (55). Moreover, mold spores possess the ability to intracellularly germinate within phagocytes (56). Certain resting spores (e.g., spores of *A. fumigatus* or *Lomentospora prolificans*) have a hydrophobic and immunologically inert outer layer which hampers the activation of the phagocyte; however, this outer layer slowly degenerates when germination starts, and as soon as phagocytes recognize fungal antigenic cell wall structures (e.g., 1,3-β-D-glucan [BDG]) during germination, they initiate the inflammatory cascade (9, 33, 56-58). Nevertheless, the success in full intracellular germination is promoted by glucocorticoids, which can impair the ability of macrophages to destroy the germinating phagocytosed spores which then continue to germinate and in turn destroy the macrophages by the developing mycelium (59, 60). Additionally, spores that are not cleared from alveoli or are not phagocytosed can initiate endocytosis into type II pneumocytes by their own (e.g., *A. fumigatus* spores) (61, 62). Most of them will be destroyed by the pneumocytes via lysosomes but a few percent of spores will not be eliminated. Although germination of the surviving intracellular spores is delayed, some of them will finally manage to germinate and grow out of the pneumocyte.

After germination of spores into hyphae, the most important line of defense are neutrophils (33, 39, 60). Neutrophils are recruited to the site of infection by cysteine-X-cysteine motif chemokine ligand (CXCL) 1 and interleukin (IL) 8, and are activated by tumor necrosis factor (TNF) α, IL-17A, and IL-18 (33, 38).

In early IMIs, these cytokines are primarily secreted by macrophages, natural killer cells (NKC), dendritic cells (DC), and innate lymphoid cells (ILC) 3, respectively, along with

interferon (IFN) γ (33, 38, 63). Immune cells damage hyphae by respiratory bursts and degranulation of non-oxidative enzymes (50, 64). Furthermore, neutrophil extracellular traps (i.e., a combination of nuclear deoxyribonucleic acid [DNA], fungicidal proteins of lysed neutrophils, and soluble PRRs like pentraxin 3) inhibit further hyphal growth (52, 65, 66). Monocytes/macrophages and NKCs are also able to neutralize hyphae but neutrophiles are more active and effective at this (64, 67). However, glucocorticoids dose-dependently reduce efficacy of cytotoxic mechanisms towards hyphae (12, 41, 68). Moreover, glucocorticoids can accelerate the metabolism of hyphae and thus cause faster hyphal growth, as demonstrated for hydrocortisone and *A. fumigatus* (68, 69).

During invasive growth, molds damage the endothelial cell layer when they break into blood vessels, causing thrombosis (61). After contact with blood, thrombocytes adhere to spores and hyphae, degranulate, and by this inhibit germination of spores and damage hyphae (70, 71). Furthermore, they enhance the fungicidal effects of neutrophils (33). Nevertheless, fungal germlings can disseminate and invade other organs after invading the bloodstream.

Interleukin 8. The main function of IL-8 (synonym: CXCL8) is to recruit neutrophils from the bloodstream into the tissue (38, 72). Together with CXCL1, both represent the most important chemokines for neutrophils (38). In addition to the chemotactic properties, IL-8 stimulates phagocytosis and degranulation by neutrophils (49, 73). IL-8 is produced by several different cell types (e.g., macrophages, vascular endothelial cells, epithelial cells, and also by neutrophils itself), with tissue macrophages being a particularly important source in infections (38, 42, 74, 75).

In vitro experiments demonstrated that hyphae, spores, enzymes, and metabolites from molds can stimulate IL-8 production in different cell types (see Table 1) (42, 53, 54, 76-79). In studies in vivo, Chai et al. (80) demonstrated that initially high or persistent IL-8 concentration in serum were predictors for treatment failure and mortality in IA. Our research group demonstrated that the concentrations of IL-8 in serum and BALF differed between cases with invasive pulmonary aspergillosis (IPA) and cases with non-*Aspergillus* pneumonia (81, 82). The discriminatory capability was acceptable to excellent (81, 82). Similarly, Goncalves et al. (83) found that IL-8 in BALF was the best factor for discriminating IPA from cases with non-*Aspergillus* pneumonia.

However, elevations of IL-8 in BALF were also associated with other pathologic processes, for example lung tissue damage in general (84).

Table 1 *Secretion of Interleukin 8 In Vitro After Contact With Molds*

Cell type (species)	Fungal morphology and species	Production of IL-8	Reference
A549 cells (human) [a]	Culture filtrate: *A. fumigatus*	↑	Tomee et al. (79)
	Opsonized spores: *A. fumigatus*	↑	Bidula, Sexton, Abdolrasouli, et al. (53); Bidula, Sexton, Yates, et al. (54).
Bronchial epithelial cells (human)	Spores: *A. fumigatus*	↑	Sun et al. (42)
Monocytes (human)	Spores: *A. fumigatus*	↑	Cortez et al. (77)
Mononuclear cells (human)	Hyphae: *R. arrhizus, R. microspores, L. corymbifera., C. bertholletiae*	↑	Roilides et al. (78)
NCI-H292 cells (human) [b]	Culture filtrate: *A. fumigatus*	↑	Tomee et al. (79)
Neutrophils (human)	Hyphae: *R. arrhizus*	↑	Chamilos et al. (76)
	Opsonized spores: *A. fumigatus*	↓	Bidula, Sexton, Abdolrasouli, et al. (53)

Note. The table illustrates the alterations in the production of interleukin (IL) 8 by the corresponding cells in experiments in vitro upon exposure to spores, hyphae, or culture filtrate of *Aspergillus fumigatus*, *Rhizopus spp.*, *Lichtheimia corymbifera*, or *Cunninghamella bertholletiae*. ↑ = significant increase; ↓ = significant decrease.

[a] A549 cells are a human epithelial cell line that represents type II pneumocytes. [b] NCI-H292 cells are a human epithelial cell line that represents bronchial epithelium.

Regarding other fungal infections, *Pneumocystis* pneumonia was also accompanied by elevated IL-8 levels in the BALF and at the same time, IL-8 was related to a higher mortality (85). IL-8 was also elevated in plasma in sepsis, especially in septic shock, and had the potential to differentiate between subsequent sepsis survivors and non-survivors (86, 87). In

acute respiratory distress syndrome (ARDS), IL-8 levels were elevated in BALF and pulmonary edema fluid, also correlating with mortality (88).

Cysteine-Cysteine Motif Chemokine Ligand 5. The main function of cysteine-cysteine motif chemokine ligand (CCL) 5 (synonym: regulated on activation, normal T cell expressed and secreted [RANTES]) is to recruit leukocytes (e.g., monocytes, NKCs, T cells), with the exception of neutrophils (38, 89). CCL5 is particularly secreted by thrombocytes, monocytes, macrophages, and NKCs, among other cells (77, 89, 90).

However, monocytes downregulated the production of CCL5 when they encountered *Aspergillus* spores (77). Similarly, the hyphae of *Rhizopus arrhizus* suppressed the secretion of CCL5 from NKCs (90). In patients with hematological malignancies who were treated with chemotherapy and subsequently developed an IFI (e.g., IA, candidiasis), serum levels of CCL5 were significantly lower on the day when the IFI was diagnosed compared to the periods before chemotherapy and after recovery from the IFIs (91). In patients who died during an IFI, CCL5 levels did not recover before death. This is congruent to another observation that low CCL5 levels in serum from patients with sepsis were associated with a poor outcome (89). In contrast, Radowsky et al. (92) reported higher CCL5 levels in serum of patients with IMIs associated with battle injuries, compared to patients with similar injuries but without IMI.

Interleukin 15. IL-15 has a homologous structure to IL-2 and thereby overlapping functions (for functions of IL-2, see chapter T Helper Cell Type 1 Response below) (38, 93, 94). In contrast to IL-2, it is mainly secreted by cells other than T cells, particularly by macrophages, but also by DCs and fibroblasts, among others (38, 94). It attracts T cells, promotes proliferation of NKCs, and enhances the ability of NKCs to damage other cells. IL-15 also helps memory cluster of differentiation (CD) 8 positive (+) (CD8+) cells to survive and proliferate (38). It activates neutrophils, which then produce more IL-8, thereby attracting even more neutrophils (38, 75, 94). However, the relevant impact of IL-15 on fungicidal mechanisms is unclear. IL-15 exhibited a positive effect on the antifungal properties of neutrophils but this appeared to be specific to the fungal species, as the IL-15-triggered fungicidal effect against *Aspergillus fumigatus* was not reproducible when neutrophils were challenged with *A. flavus* (75). In addition, the enhanced respiratory bursts in response to *Aspergillus* hyphae did not increase damage to hyphae concordantly and IL-15 rather

promoted non-oxidative defense mechanisms; a similar observation was made with *Candida albicans* (75, 94). However, diagnostic potential was considered possible for IL-15 as, for example, *C. albicans* significantly increased IL-15 expression in mononuclear cells (MNC) in vitro, and persistently higher IL-15 levels were associated with a higher risk of IMIs in immunocompetent patients after battle injuries (92, 95).

T Cell Immunity and Cytokine Profiles

Every time a pathogen challenges the host, the host must adapt its defense strategy (96). T cells are a key part of this process, working as a bridge between the innate and the adaptive immune responses (97). Some T cells primarily regulate other immune cells; other T cells directly participate in pathogen elimination. The group of T cells includes cytotoxic T lymphocytes (CTL), γδ T cells, mucosal-associated invariant T cells (MAIT), natural killer T cells (NKTC), T helper (Th) cells, and regulatory T cells (Treg) (38). In blood circulation, there are mainly Th cells and CTLs, with Th cells usually outnumbering CTLs. Next in number are NKTCs, and a low proportion are MAITs. Tregs and γδ T cells are rarely present in blood circulation.

Th cells are essential in stimulating and coordinating the different cells of the immune system (38, 96, 97). Certain stimuli (e.g., cytokine milieu present, amount of antigen, stimulation of specific PRRs, among others) can prompt naïve CD4+ T cells to proliferate and differentiate into specific Th cell subsets, which are defined by their cytokine profiles (38). These cytokine profiles initiate an immune response which can be helpful but also harmful. Because of the observation that the Th cell responses are associated with specific pathogens, elevations in certain cytokines could possibly indicate the specific causative pathogens. Moreover, a certain preexisting cytokine profile could maybe determine a host's vulnerability to a specific pathogen or could be a risk factor to develop a detrimental immune response after a specific infectious disease has been acquired.

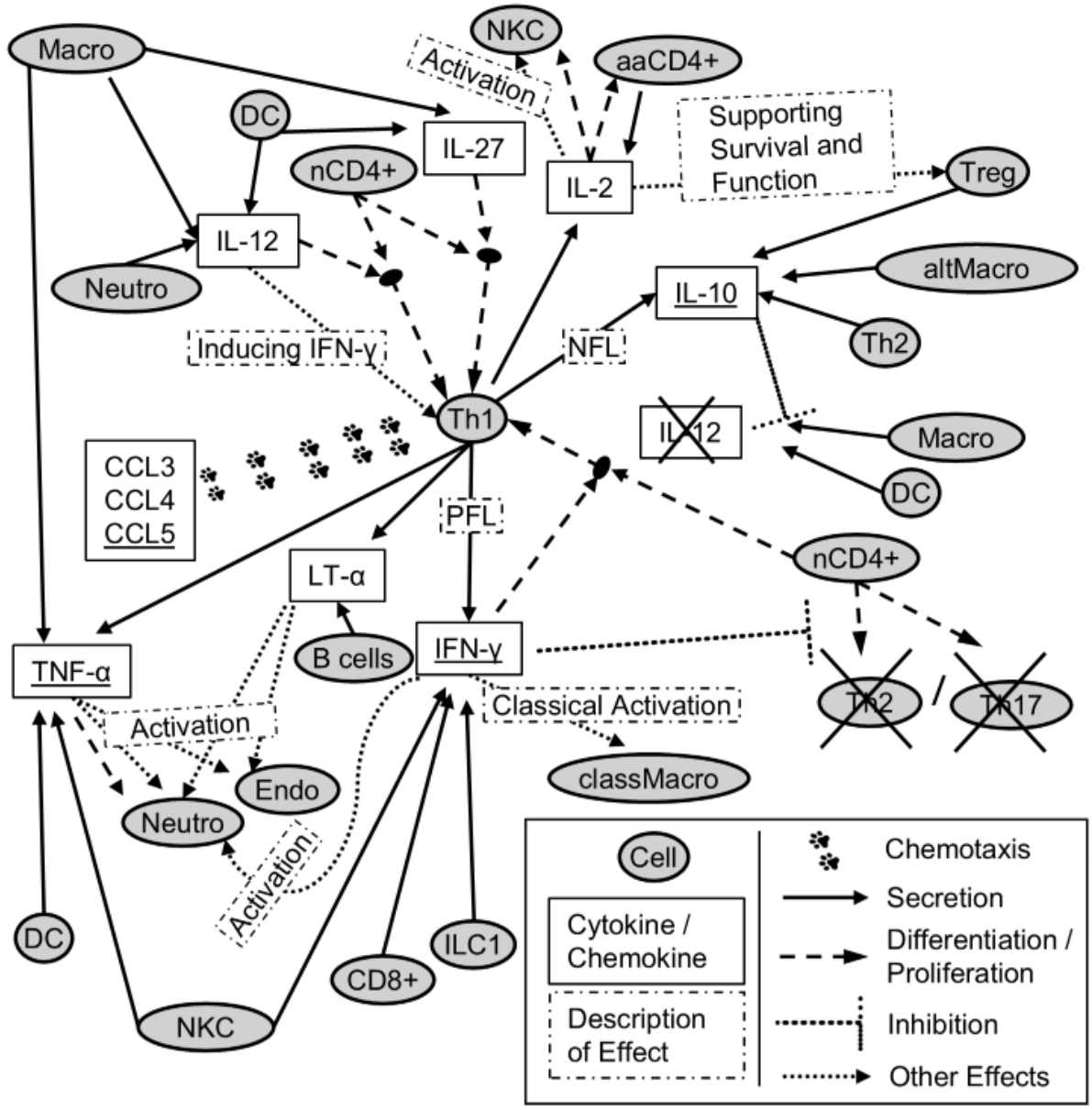
Mosmann et al. (98) first defined the T helper cell type 1 (Th1) and Th2 subsets in mice. Since then, other subsets have been defined, but Th1s and Th2s are still the most studied (97). They are antagonistically related to each other and can be roughly categorized based on their effect on the immune response: Th1s primarily promote a cellular-based phagocyte-mediated immune response and are considered as pro-inflammatory. Th2s primarily promote an antibody-based humoral immune response while they parallelly inhibit

the cellular immune response (33, 38, 93). Over time, Th0, Th3, Th9, Th17, Th22, and T follicular helper cells (Tfh) were also described as specific subsets.

T Helper Cell Type 1 Response. The Th1 response is crucial for clearing intracellular pathogens (e.g., mycobacteria, viruses) and fungi but it is also seen in autoimmune diseases, hypersensitivity reactions, and chronic inflammatory diseases (38, 93, 97, 99). IFN-γ is the signature cytokine of the Th1 response, complemented by TNF-α and lymphotoxin (LT) α (synonym: TNF-β) (50, 93). Figure 1 shows important cells, cytokines, and chemokines directly associated with the Th1 response, and their interrelationships. Th1s are vital for the so-called classical activation of macrophages via IFN-γ that (a) enhances the microbiocidal capabilities of macrophages (e.g., increasing the production of cytotoxic substances; enabling the neutralization of phagocytosed pathogens); (b) stimulates their phagocytic activity; and (c) enhances their antigen presenting capacities (38). In addition, the Th1 response promotes the formation of CTLs and inhibits the Th2 and Th17 response by cross-regulation via IFN-γ (38, 50, 93).Via cysteine-X-cysteine motif chemokine receptor (CXCR) 3 and cysteine-cysteine motif chemokine receptor (CCR) 5, Th1s are recruited to inflammatory sites by the chemokines CCL3, CCL4, and CCL5, among others (38, 100). A crucial cytokine for developing a Th1 response—and parallelly inhibiting Th2 responses—is IL-12, secreted by DCs, macrophages, and neutrophils after contact with mold antigens (38, 93, 96). Additionally, NKCs can also initiate Th1 differentiation via IFN-γ (38, 97). In fact, IFN-γ also plays a key role for differentiation of Th1s, forming a positive feedback loop for further Th1 subset differentiation. For autoregulatory purposes, Th1s also secrete IL-10.

IL-2 is a key cytokine for the clonal proliferation of antigen-specific CD4+ and CD8+ T cells, and is secreted autocrinally when naïve CD4+ or CD8+ T cells have been activated by antigen presentation (38). However, it is also expressed by differentiated Th1s which by this way additionally support the inflammatory reaction (38, 97). IL-2 allows the rapid generation of many antigen-specific T cell clones. Furthermore, it also sustains the survival of T cells. Apart from the proliferation of CD4+ and CD8+ T cells, IL-2 promotes NKC proliferation and activation, but also fulfills an autoregulatory role on inflammation by supporting survival and functions of Tregs.

Figure 1 *Cytokines and Chemokines Associated With a T Helper Cell Type 1 Response*



Note. This simplified illustration delineates the principal effects and primary sources of cytokines that are characteristic of T helper cells type (Th) 1 (i.e., interferon [IFN] γ, lymphotoxin [LT] α, and tumor necrosis factor [TNF] α). Furthermore, cytokines that are pivotal for the development of Th1s are depicted, along with a selection of major chemokines for Th1s. The Th1 response includes a positive feedback loop (PFL) via IFN-γ and a negative feedback loop (NFL) via interleukin (IL) 10. The cytokines and chemokines evaluated in this thesis are indicated with underscoring. The underlying information for this figure was derived from Abbas et al. (38); Heimall (97); Jung & Littman (100); Lucey et al. (93); and Romani (50). aaCD4+ = antigen-activated cluster of differentiation 4 positive T cell;

altMacro = alternatively activated macrophage; CCL = cysteine-cysteine motif chemokine ligand; CD8+ = cluster of differentiation 8 positive T cell; classMacro = classically activated macrophage; DC = dendritic cell; Endo = endothelial cell; ILC = innate lymphoid cell; Macro = macrophage; nCD4+ = naïve cluster of differentiation 4 positive T cell; Neutro = neutrophil; NKC = natural killer cell; Treg = regulatory T cell.

Interferon γ. IFN-γ has a pro-inflammatory effect on immune responses and is the main recruiting and activating cytokine for macrophages (38, 50, 101, 102). Besides the classical activation of macrophages, it also stimulates the phagocytic activity of neutrophils as well as increases the cytotoxic abilities of both neutrophils and NKCs (38, 49, 50, 101-103). Additionally, it has a directly damaging effect on germinating spores of *Aspergillus fumigatus* (49, 102-104). In general, IFN-γ is considered protective in IA (105).

T cells (particularly Th1s and CD8+ T cells) and NKCs are major sources of IFN-γ (38, 72, 93). For example, during the early phase of IPA in neutropenic mice, NKCs were the main source of IFN-γ in lungs (63). ILC1s also enhance the Th1 response via IFN-γ production (38). ILC1s develop from their corresponding precursor cells under the influence of IL-7 and IL-15 and are activated under the influence of IL-12 and IL-18 (38). It was demonstrated that certain human and mouse cells increased production of IFN-γ when stimulated with mold spores or hyphae (see Table 2) (67, 90, 106-110). However, the hyphae of *A. fumigatus* and *Rhizopus arrhizus* were able to inhibit IFN-γ secretion by human NKCs (67, 90). Nevertheless, *Aspergillus*-specific T cells that produced IFN-γ were demonstrated in patients with IA (111). Similarly, CD4+ T cells produced IFN-γ in mice after *A. fumigatus* spores were injected intravenously, causing measurable elevated IFN-γ levels in serum (112). These elevated IFN-γ levels were also associated with survival and subsequent immunity of mice to IA. In addition, elevated IFN-γ levels were demonstrated in the BALF of mice challenged with spores of *A. fumigatus* (109, 113). However, treatment with cyclophosphamide or neutropenia led to decreased production of IFN-γ in the lungs of mice (106). Moreover, some mycotoxins produced by molds can shift T cell cytokine profiles to Th2 patterns, thus inhibiting IFN-γ production (114).

Table 2 *Secretion of Interferon γ In Vitro After Contact With Molds*

Cell type (species)	Fungal morphology and species	Production of IFN-γ	Reference
CD4+ T cells (mouse)	Hyphae: *A. fumigatus*	↑	Rivera et al. (109)
Lymphocytes (mouse)	Spores: *A. fumigatus*	↑	Cenci et al. (106)
Macrophages/lymphocytes system (human)	Spores: *A. fumigatus*	↑	Chai et al. (107)
Mononuclear cells (human)	Spores: *A. fumigatus*	↑	Grazziutti et al. (108); Gresnigt et al. (110)
Natural killer cells (human)	Hyphae: *A. fumigatus, R. arrhizus*	↓	Schmidt, Tramsen, Hanisch et al. (67); Schmidt, Tramsen, Perkhofer et al. (90)
	Germinating Spores: *A. fumigatus*	↓	Schmidt, Tramsen, Hanisch et al. (67)
	Resting Spores: *A. fumigatus, R. arrhizus*	→	Schmidt, Tramsen, Hanisch et al. (67); Schmidt, Tramsen, Perkhofer et al. (90)

Note. The table illustrates the alterations in the production of interferon (IFN) γ by the corresponding cells in experiments in vitro upon exposure to spores or hyphae of *Aspergillus fumigatus* or *Rhizopus arrhizus*. → = no significant change; ↑ = significant increase; ↓ = significant decrease; CD4+ = cluster of differentiation 4 positive.

Tumor Necrosis Factor α. TNF-α plays a critical role in activating neutrophils, enhancing their respiratory burst and degranulation, and promoting their proliferation in bone marrow (38, 72, 115). It also stimulates endothelial cells to produce chemokines and adhesion molecules, leading to additional migration of leukocytes to the site of infection (38, 116). However, coagulation is also triggered and is harmful to the host when followed by thrombosis (38). Nevertheless, the overall effect of TNF-α is generally considered to be protective in IA (105).

Macrophages, DCs, NKCs, and Th1s are major sources of TNF-α (38, 116). However, other cells, such as B cells, bronchial epithelial cells, endothelial cells, and monocytes, can also produce TNF-α (42, 74, 117). Except for granulocytes, TNF-α production increased in various human and mouse cells exposed to hyphae in vitro (see Table 3) (74, 78, 109, 117-121). Particularly hyphae of *R. arrhizus* were effective at triggering TNF-α production in MNCs, followed by *Cunninghamella bertholletiae* (78). In contrast, spores induced less effectively TNF-α production, excepting spores of *Lomentospora prolificans* (74, 118-121).

TNF-α levels were elevated in the lung tissue and BALF of immunocompetent mice that became infected after instillation of *A. fumigatus* spores (113, 122, 123). However, elevated TNF-α serum levels were not detect in non-neutropenic patients with IA, according to Roilides et al. (124). Similarly, Goncalves et al. (83) found no elevated TNF-α concentrations in serum from patients with IPA, compared to those with non-*Aspergillus* pneumonia, although they found different TNF-α concentrations in BALF between the two groups.

TNF-α is crucial for the function of the immune system: Mice with neutralized TNF-α were more susceptible to IA, experienced increased fungal growth, and had worse outcomes (72, 122). Similarly, treatment with TNF-α blockers raised the risk of opportunistic infections including IMIs in humans (125-127). Apart from TNF-α blockers, other immunosuppressants can also inhibit TNF-α responses. For example, glucocorticoids reduced TNF-α production of murine alveolar macrophages (128). Consistent with this, TNF-α was not measurable in BALF of mice with IPA and glucocorticoid-induced immunosuppression, while it was elevated in neutropenic mice with IPA but without concomitant glucocorticoid administration (122, 123). Nevertheless, TNF-α levels were generally lower in immunocompromised mice than in immunocompetent (106). Similar to mice, glucocorticoids inhibited TNF-α secretion of human monocytes in vitro, despite contact with *Candida albicans* (129). Complicating the evaluation of diagnostic potential of TNF-α concentrations, TNF-α secretion is unspecific and is not exclusively triggered by molds or yeasts. For example, *Pneumocystis* also increased TNF-α levels in the BALF of mice; and in humans, bacterial community-acquired pneumonia (CAP) or ARDS of various etiologies were also associated with elevated TNF-α in BALF (130-132).

Table 3 *Secretion of Tumor Necrosis Factor α In Vitro After Contact With Molds*

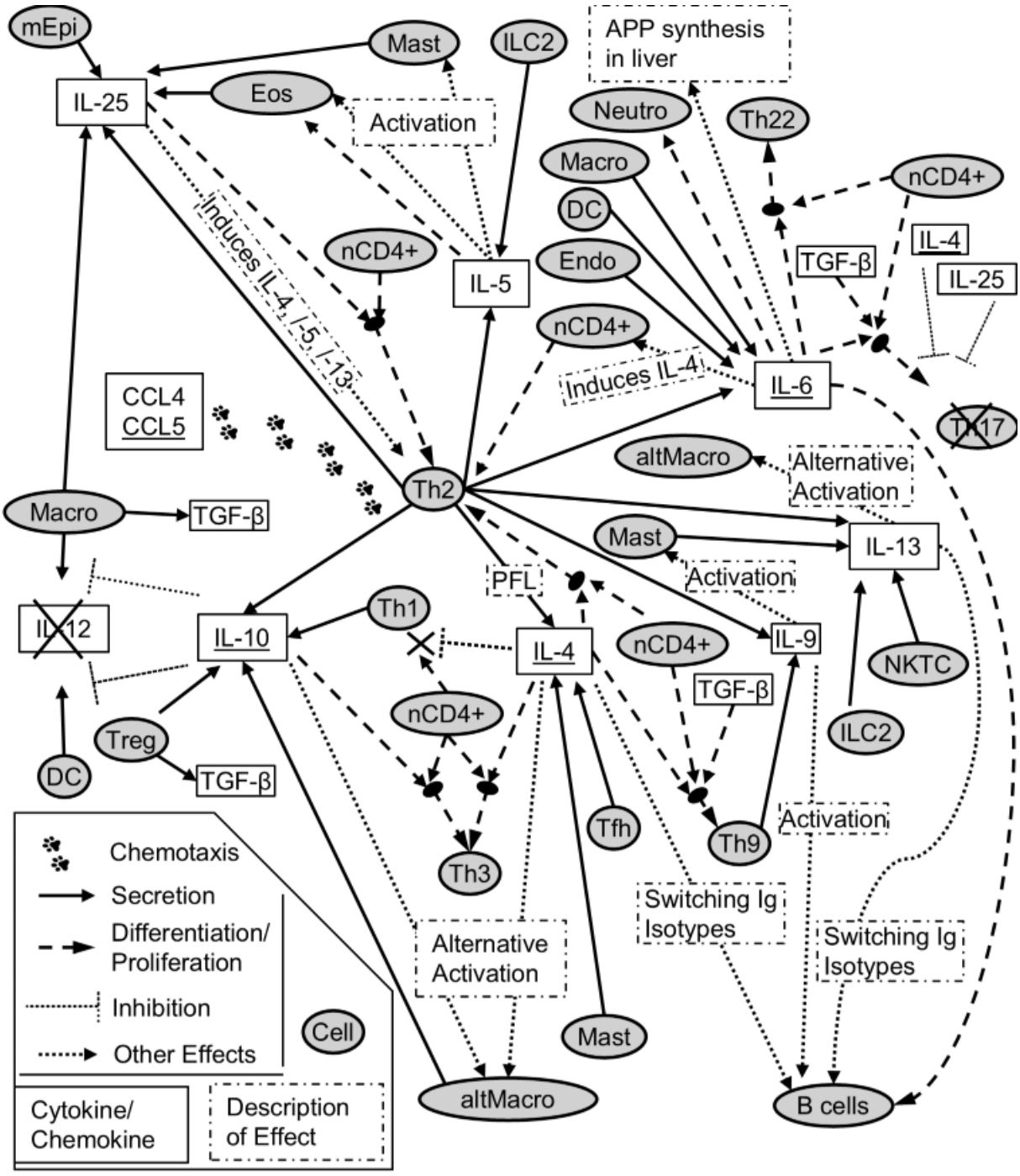
Cell type (species)	Fungal morphology and species	Production of TNF-α	Reference
B cells (human)	Hyphae: *A. fumigatus, L. prolificans*	↑	Wang et al. (117)
Bronchial epithelial cells (human)	Spores: *A. fumigatus*	↑	Sun et al. (42)
CD4+ T cells (mouse)	Hyphae: *A. fumigatus*	↑	Rivera et al. (109)
Dendritic cells (human)	Hyphae: *A. fumigatus, R. arrhizus*	↑	Chamilos et al. (119)
	Spores: *A. fumigatus, R. arrhizus*	→	Chamilos et al. (119)
Dendritic cells (mouse)	Hyphae: *A. fumigatus*	↑	Bozza et al. (118)
	Spores: *A. fumigatus*	↑	Bozza et al. (118)
Endothelial cells (human)	Hyphae: *A. fumigatus*	↑	Chiang et al. (74)
	Spores: *A. fumigatus*	→	Chiang et al. (74)
Granulocytes (human)	Hyphae: *A. fumigatus, L. prolificans*	→	Wang et al. (117)
Monocytes (human)	Hyphae: *A. fumigatus, L. prolificans*	↑	Wang et al. (117)
	Spores: *A. fumigatus, A. flavus, A. terreus, A. niger; R. arrhizus*	↑	Warris, Netea, Verweij et al. (121)
Monocytes (human)	Spores: *L. prolificans*	↑ ↑	Warris, Netea, Verweij et al. (121)
Mononuclear cell (human)	Hyphae: *A. fumigatus, R. microspores, L. corymbifera, C. bertholletiae*	↑	Roilides et al. (78); Warris, Netea, Verweij et al. (121)
	Hyphae: *R. arrhizus*	↑ ↑	Roilides et al. (78)
	Spores: *A. fumigatus*	↑	Grazziutti et al. (108)

Cell type (species)	Fungal morphology and species	Production of TNF-α	Reference
T cells (human)	Hyphae: *A. fumigatus, L. prolificans*	↑	Wang et al. (117)
Whole blood (human)	Hyphae: *A. fumigatus*	↑	Warris, Netea, Wang et al. (120)
	Spores: *A. fumigatus*	↑	Warris, Netea, Wang et al. (120)

Note. The table illustrates the alterations in the production of tumor necrosis factor (TNF) α by the corresponding cells in experiments in vitro upon exposure to spores or hyphae of *Aspergillus spp.*, *Rhizopus spp.*, *Lomentospora prolificans*, *Lichtheimia corymbifera*, and *Cunninghamella bertholletiae*. → = no significant change; ↑ = significant increase; ↑↑ = significant and particularly marked increase; CD4+ = cluster of differentiation 4 positive.

T Helper Cell Type 2 Response. Th2s primarily support antibody-based humoral immune responses and inhibit cellular-based immune response (33, 93). They can also contribute to allergic diseases (38, 50, 96). One example is allergic bronchopulmonary aspergillosis (ABPA) in which a persistent Th2 response in blood and BALF is triggered (133, 134). Signature cytokines of the Th2 subset are IL-4, IL-5, and IL-13 (38, 93, 97). Other characteristic cytokines include IL-6, IL-9, and IL-10. Figure 2 shows important cells, cytokines, and chemokines directly associated with the Th2 response, and their interrelationships. The Th2 response stimulates the proliferation and activation of eosinophils and mast cells, which are important in defense against parasites (38, 50, 96). Additionally, B cells are activated and stimulated, leading them to switch antibody isotypes to immunoglobulin (Ig) G4 and IgE (96). IL-4 and IL-10 activate macrophages via the alternative pathway, by this inhibiting microbiocidal and inflammation-inducing functions of macrophages but in turn activating their tissue repairing mechanisms (e.g., inducing fibrotic processes) (38, 93). However, IL-4 did not impair clearance of phagocytosed spores in vitro (135); similarly, IL-10 did not affect intracellular sporicidal activity, and phagocytic activity against spores was actually upregulated (49, 136).

Figure 2 *Cytokines and Chemokines Associated With a T Helper Cell Type 2 Response*



Note. This simplified illustration delineates the principal effects and primary sources of cytokines that are characteristic of T helper cells type (Th) 2 (i.e., interleukin [IL] 4, IL-5, IL-6, IL-9, IL-10, IL-13). Furthermore, cytokines that are pivotal for the development of Th2s are shown, along with a selection of chemokines. The Th2 response includes a positive feedback loop (PFL) via IL-4. The cytokines and chemokines evaluated in this thesis are indicated with underscoring. The underlying information for creation of this figure was

derived from Abbas et al. (38); Heimall (97); Jung & Littman (100); Lucey et al. (93); Romani (50); Romani et al. (96); and Scheller et al. (137). altMacro = alternatively activated macrophage; APP = acute-phase proteins; CCL = cysteine-cysteine motif chemokine ligand; DC = dendritic cell; Endo = endothelial cell; Eos = eosinophil; Ig = immunoglobulin; ILC = innate lymphoid cell; Macro = macrophage; Mast = mast cell; mEpi = mucosal epithelial cell; nCD4+ = naïve cluster of differentiation 4 positive T cell; Neutro = neutrophil; NKTC = natural killer T cell; Tfh = T follicular helper cell; TGF = transforming growth factor; Treg = regulatory T cell.

Overall, however, activating macrophages via the alternative pathway is considered less protective against fungi than activation via the classical pathway (38, 49, 50).

Th2s express several chemokine receptors, including CCR3, CCR4, CCR8, and CCR10, which attract them to inflammatory sites via several chemokines, including CCL4 and CCL5 (38, 97, 100). IL-4 and IL-10 inhibit the Th1 response, while IL-4 and IL-25 promote the differentiation of naïve CD4+ T cells to Th2s; in this way, IL-4 serves as an important positive feedback mechanism of the Th2 response (38, 93).

Interleukin 4. IL-4 promotes the proliferation and differentiation of naïve CD4+ T cells into Th2s, while it inhibits the Th1 response and the differentiation of Th17 subsets (38). It is particularly produced by T cells (viz., Th2s and Tfhs) and mast cells (38, 93, 138). Generally, IL-4 is considered a debilitating factor in IA. The inhibition of IL-4 positively affected the curse of IA in mice, whereas IL-4 production was associated with an exacerbation of IA (106, 112). After an intravenous injection of *Aspergillus fumigatus* spores, Cenci et al. (112) observed increased IL-4 production from CD4+ T cells of mice. Moreover, the CD4+ T cells from mice that died from the infection secreted more IL-4 in vitro than CD4+ T cells from mice that survived the infection. However, increased IL-4 levels were not detectable in serum of both groups of mice. For IPA in immunocompetent mice, there are conflicting results whether IL-4-secreting lymphocytes accumulate in the lungs, whereas it was demonstrated that such lymphocytes accumulated in lungs of immunocompromised mice (106, 109). Regarding humans, MNCs obtained from peripheral blood of healthy probands could not be stimulated by inactivated *A. fumigatus* spores to produce IL-4 in vitro (108). In patients with hematological malignancies and IA, *Aspergillus*-specific T cells that produced IL-4 were less frequent than those producing IL-10 or IFN-γ (111). However, mycotoxins

from *A. fumigatus* were able to shift Th1 cytokine patterns to Th2 patterns, leading to a proportional increase of IL-4 production (114).

Interleukin 6. IL-1, TNF-α, and IL-6 are important pro-inflammatory cytokines of the innate immune system (38). IL-6 is involved in T cell differentiation and proliferation, for example, in differentiation of naïve CD4+ T cells into Th2s or Th17s (38, 105, 137). In hepatocytes, IL-6 stimulates production of acute phase proteins such as C-reactive protein (CRP), which is a pentraxin and can activate the classical complement pathway after binding to fungi (38, 139). In hematopoiesis, IL-6 leads to the proliferation of multipotential progenitor cells and stimulates neutrophil production (38, 139, 140). Overall, IL-6 is considered to be protective in IA (105). T cells, monocytes/macrophages, and endothelial cells are major sources of IL-6 (38, 72, 93). Furthermore, IL-6 is generally released from a variety of other cells (e.g., DCs, stromal cells, parenchymal cells) subsequently to tissue damage. It promotes various effects besides its influence on the immune response (38, 72, 140). Various cells from mice and humans produced higher amounts of IL-6 upon contact with mold spores or hyphae, particularly after contact with hyphae of *Rhizopus arrhizus* (see Table 4) (53, 54, 78, 79, 109, 119-121, 141). However, glucocorticoids decreased production of IL-6 (139, 142, 143).

Mice with IPAs had elevated IL-6 levels in BALF, with higher levels in immunocompromised than in immunocompetent mice (106, 109). In humans, our research group found higher IL-6 levels in serum and BALF in cases of IPA than in cases of non-*Aspergillus* pneumonia (81, 82). This was later confirmed by Goncalves et al. (83) for BALF. Chai et al. (80) demonstrated that non-decreasing levels of IL-6 in serum were associated with treatment failure of IA and higher mortality. Similarly, elevated IL-6 concentrations were associated with higher mortality in sepsis due to bacterial infections (144). However, IL-6 was elevated in non-septic infections as well, for example in BALF and serum in cases of bacterial CAP (131).

Interleukin 10. IL-10 plays an important role in regulating the inflammatory responses and is produced by many cells, but especially by alternatively activated macrophages and T cells, among other cells (38, 50, 72, 145).

Table 4 *Secretion of Interleukin 6 In Vitro After Contact With Molds*

Cell type (species)	Fungal morphology and species	Production of IL-6	Reference
A549 cells (human) [a]	Culture filtrate: *A. fumigatus*	↑	Tomee et al. (79)
	Hyphae: *A. fumigatus*	↑	Zhang et al. (141)
	Spores: *A. fumigatus*	↑	Zhang et al. (141)
	Opsonized spores: *A. fumigatus*	↓/→	Bidula, Sexton, Abdolrasouli, et al. (53); Bidula, Sexton, Yates, et al. (54)
CD4+ T cells (mouse)	Hyphae: *A. fumigatus*	↑	Rivera et al. (109)
Dendritic cells (human)	Hyphae: *A. fumigatus, R. arrhizus*	↑	Chamilos et al. (119)
Monocytes (human)	Spores: *L. prolificans*	↑	Warris, Netea, Verweij et al. (121)
	Spores: *A. fumigatus, A. flavus, A. terreus, A. niger, R. arrhizus*	→	Warris, Netea, Verweij et al. (121)
Macrophages/lymphocytes system (human)	Spores: *A. fumigatus*	↑	Chai et al. (107)
Mononuclear cells (human)	Hyphae: *A. fumigatus*	↑	Roilides et al. (78)
	Hyphae: *R. arrhizus*	↑↑	Roilides et al. (78)
NCI-H292 cells (human) [b]	Culture filtrate: *A. fumigatus*	↑	Tomee et al. (79)
Whole blood (human)	Hyphae: *A. fumigatus*	↑	Warris, Netea, Wang et al. (120)
	Spores: *A. fumigatus*	↑	Warris, Netea, Wang et al. (120)

Note. The table illustrates the alterations in the production of interleukin (IL) 6 by the corresponding cells in experiments in vitro upon exposure to spores, hyphae, or culture filtrate of *Aspergillus spp.*, *Rhizopus arrhizus*, or *Lomentospora prolificans*. → = no significant change; ↑ = significant increase; ↑↑ = significant and particularly marked increase;

↓ = significant decrease; CD4+ = cluster of differentiation 4 positive.

[a] A549 cells are a human epithelial cell line that represents type II pneumocytes. [b] NCI-H292 cells are a human epithelial cell line that represents bronchial epithelium.

Among T cells, IL-10 is not only produced by Th2s, but also by, for example, Th1s, representing a negative feedback mechanism to reduce production of pro-inflammatory cytokines and tissue damage (38, 50, 146). However, the dominant production of IL-10 is thought to promote the progression of IA, particularly during its early stages (49, 50, 105). This is supported by experiments in which mice with impaired immune systems showed improved outcomes when IL-10 was inhibited (106). However, glucocorticoids are considered to more severely attenuate the antifungal activity of phagocytes than IL-10 (135).

In vitro experiments demonstrated that particularly hyphae stimulated IL-10 production, whereas experiments with spores produced inconsistent results (see Table 5) (77, 108, 109, 120, 121). Furthermore, *Aspergillus fumigatus* spores injected into the bloodstream of mice promoted CD4+ T cells that produced IL-10 (112). However, this did not lead to increased IL-10 levels in serum. Contrary, increased IL-10 concentrations in BALF that at least partly came from CD4+ T cells were demonstrated in immunosuppressed mice with IPA (106, 109, 123). Interestingly, bronchoalveolar IL-10 levels were low or undetectable in immunocompetent mice in *A. fumigatus* infections. In patients with cystic fibrosis and *A. fumigatus*-associated lung diseases, levels of IL-10 were elevated in serum (147). Likewise, Roilides et al. (124) found higher initial IL-10 levels in serum of non-neutropenic IA patients than of healthy control participants. In the further process of the study, IL-10 levels remained low or decreased in IA cases without progression, whereas progression was associated with increasing IL-10 levels. Similar observations were made with other infections: In severe bacterial infections, IL-10 levels had a prognostic value, with higher IL-10 levels being associated with higher mortality and morbidity (147). However, the published data for prognosis of IA is contradictory, as Chai et al. (80) associated higher IL-10 levels with lower mortality, for example.

T Helper Cell Type 17 Response. The Th17 response is a pro-inflammatory response that is important for recruiting and activating neutrophils and monocytes/macrophages, which are crucial for defense against extracellular bacteria and fungi (38).

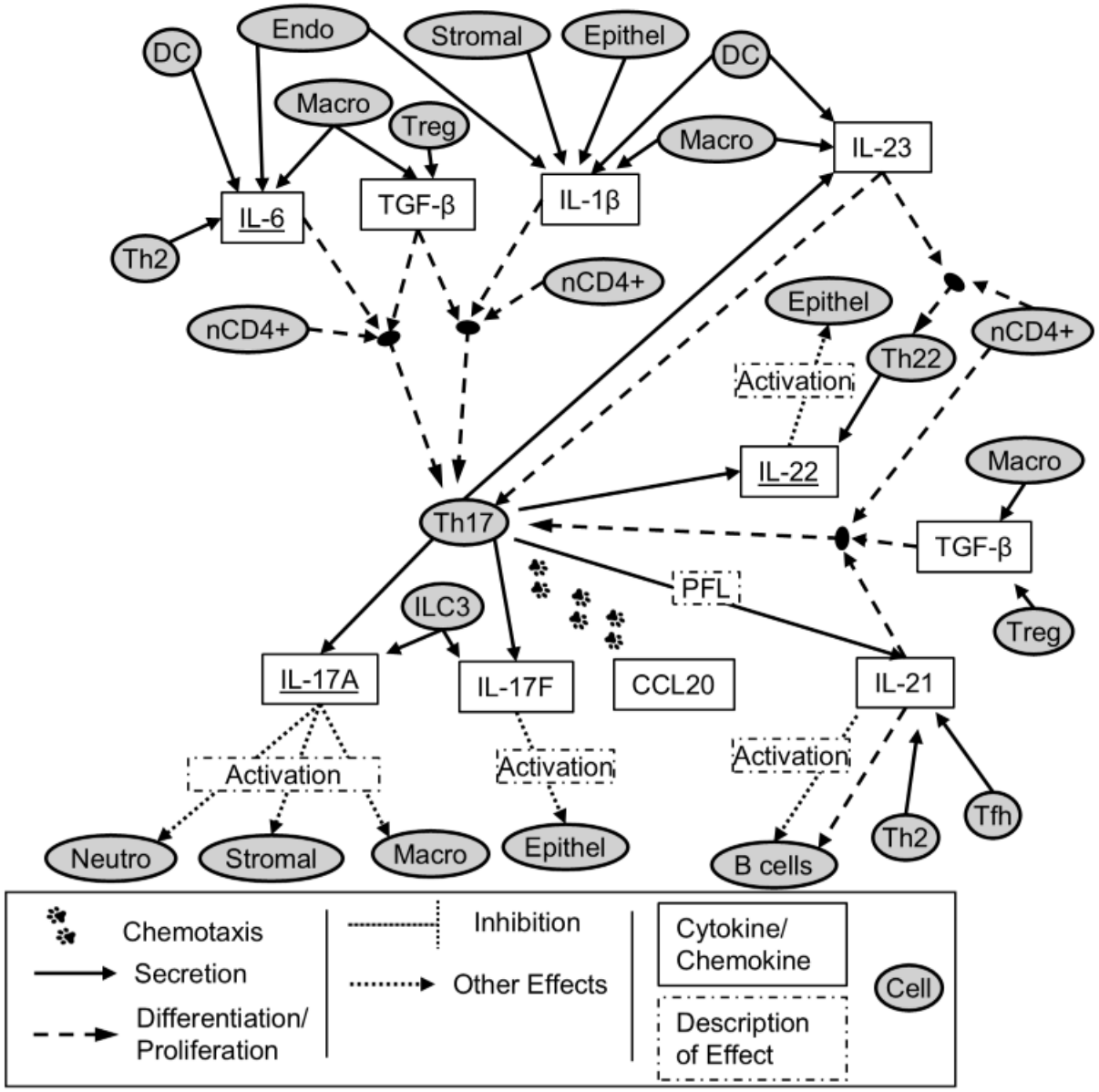
Table 5 *Secretion of Interleukin 10 In Vitro After Contact With Molds*

Cell type (species)	Fungal morphology and species	Production of IL-10	Reference
CD4+ T cells (mouse)	Hyphae: *A. fumigatus*	↑	Rivera et al. (109)
Monocytes (human)	Hyphae: *A. fumigatus*	↑	Warris, Netea, Verweij et al. (121)
	Spores: *A. fumigatus*	↑	Cortez et al. (77)
	Spores: *A. fumigatus*	→	Warris, Netea, Verweij et al. (121)
Mononuclear cells (human)	Spores: *A. fumigatus*	→	Grazziutti et al. (108)
Whole blood (human)	Hyphae: *A. fumigatus*	↑	Warris, Netea, Wang et al. (120)
	Spores: *A. fumigatus*	↑	Warris, Netea, Wang et al. (120)

Note. The table illustrates the alterations in the production of interleukin (IL) 10 of the corresponding cells in experiments in vitro upon exposure to *Aspergillus fumigatus* spores or hyphae. → = no significant change; ↑ = significant increase; CD4+ = cluster of differentiation 4 positive.

It was demonstrated that *Candida albicans* generated a potent Th17 response in vitro, for example (148). However, the induction and relevance of Th17 responses in aspergillosis is controversially discussed, as *A. fumigatus* produces kynurenine which is inhibitory for Th17 cytokine production (107). The signature cytokine of Th17s is IL-17A, which is responsible for most immunologic effects of the IL-17 cytokine family and also represents the most important member of the IL-17 family for defending bacterial and fungal infections (38, 149). The IL-17 cytokine family consists of six structurally related proteins, with IL-17F being the structurally most similar one to IL-17A. However, IL-17F mainly plays a role in the defense mechanisms of mucosal surfaces (149). The relevance of IL-17B, IL-17C, and IL-17D for the immune response is largely unknown. IL-17E and IL-25 are synonyms, and this cytokine has the least structural similarity to IL-17A, is primarily observed in the Th2 response, and inhibits the development of Th17s. Apart from IL-17A and IL-17F, further characteristic cytokines of Th17s are IL-21 and IL-22 (38, 149). Figure 3 shows important cells, cytokines, and chemokines directly associated with the Th17 response, and their interrelationships.

Figure 3 *Cytokines and Chemokines Associated With a T Helper Cell Type 17 Response*



Note. This simplified illustration delineates the principal effects and primary sources of cytokines that are characteristic of the T helper cells type (Th) 17 (i.e., interleukin [IL] 17A, IL-17F, IL-21, IL-22). Furthermore, the cytokines that are pivotal for the development of Th17s are depicted, along with cysteine-cysteine motif chemokine ligand (CCL) 20, a pivotal chemokine for Th17s. The Th17 response includes a positive feedback loop (PFL) via IL-21. The cytokines and chemokines evaluated in this thesis are indicated with underscoring. The underlying information for this figure was derived from Abbas et al. (38); Bettelli et al. (150); Heimall (97); Iwakura et al. (149); Jung & Littman (100); and Scheller et al. (137).
DC = dendritic cell; Endo = endothelial cell; Epithel = epithelial cell; ILC = innate lymphoid

cell; Macro = macrophage; nCD4+ = naïve cluster of differentiation 4 positive T cell; Neutro = neutrophil; Stromal = stromal cells; Tfh = T follicular helper cell; TGF = transforming growth factor; Treg = regulatory T cell.

Th17s express CCR6 that is the receptor for CCL20, a chemokine produced by macrophages and tissue cells challenged with bacterial or fungal infections (38, 100). Transforming growth factor (TGF) β combined with (a) IL-1β, (b) IL-21, or (c) IL-6 plus soluble interleukin 6 receptor (sIL-6R) induces the differentiation of Th17s from naïve CD4+ T cells (38, 97, 137, 150). In contrast, TGF-β on its own would promote the differentiation of naïve CD4+ T cells to Tregs, and IL-6 on its own would stimulate IL-4 formation in the naïve CD4+ T cells and by this promoting differentiation to Th2s (137). IL-21 is part of a positive feedback mechanism (38, 97, 150). The Th17 differentiation is inhibited by Th1 or Th2 responses via IFN-γ or IL-4, respectively (38). After the differentiation of Th17s has been initiated, IL-23 is important for further stabilization and proliferation.

Interleukin 17A. IL-17A induces the production of various chemokines (e.g., IL-8) and cytokines (e.g., IL-6) in epithelial cells, macrophages, and others (38, 151). It also increases the production of antimicrobial peptides like defensins (38, 151). At the same time, it increases the synthesis of granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), leading to proliferation and migration of neutrophils and monocytes (38, 152). However, IL-17 was rather detrimental in mice with IA: The antifungal response of neutrophils was less effective in fungal clearance and the inflammatory response failed to resolve (153).

IL-17A is particularly produced by Th17s and ILC3s, among others (38, 152). In vitro, it was demonstrated that various bacteria and *Candida albicans* induced IL-17A production in MNCs (148). Krause et al. (154) demonstrated that IL-17A levels in serum had a diagnostic value for candidemia, with potential to distinguish candidemia from other infections in severely ill patients. In another assessment, however, IL-17A plasma concentrations were elevated in both groups of patients with candidemia or bacteremia, respectively, but without potential for differentiating candidemia and bacteremia (155, 156).

However, inactivated spores and hyphae of *Aspergillus fumigatus* only weakly induced IL-17A in human MNCs in vitro (107, 157). In patients with IA, *Aspergillus*-specific T cells produced IL-10 or IFN-γ more often than IL-17A (111). Similarly, Chai et al. (107)

found only low IL-17A levels in serum and BALF from patients with hematological malignancies and IA. Surprisingly, their patients with IA had lower serum IL-17A levels than patients without IA, while there was no difference between the groups in BALF. In contrast, Goncalves et al. (83) reported higher IL-17A concentrations in serum and BALF from patients with IA in comparison to other pulmonary infections.

Interleukin 22. IL-22 particularly affects the epithelial cells and fibroblasts (38). It promotes tissue repair processes and stimulates the production of chemokines and antimicrobial peptides in epithelial cells (38, 158). IL-22 is produced particularly by Th17s, but also by other T cells, ILC3s, and NKCs, among others (38, 157, 159).

In mice with IPA, IL-22 had a protective effect, as blocking the cytokine or a deficient production caused IPAs to exacerbate (160, 161). In humans, it was demonstrated that *Aspergillus* stimulated IL-22 production in various cell types, including Th cell subtypes (110, 157).

Other T Cells as Sources of Cytokines. The major cytokine of the Tfh subset is IL-21, but Tfhs may also produce IL-4 or IFN-γ in significant amounts (38). However, Tfhs migrate into lymphoid follicles and germinal centers after their differentiation, unlike Th1s, Th2s, and Th17s which migrate to the site of infection. After approaching lymphoid follicles, Tfhs take part in generating germinal center and in activating and differentiating B cells, and by this influencing the host's antibody production (38). Th0s predominantly produce both IL-4 and IFN-γ (162). Major cytokines of Th9s and Th22s are IL-9 or IL-22, respectively (38, 97). However, it is still under discussion if these cells form separate Th subsets or are merely intermediate stages during the ongoing differentiation process (38). CTLs, which express CD8, are commonly the second most frequent T cells in blood circulation and aim for killing infected cells of the host (e.g., infected by viruses) or tumor cells, and in this process also release IFN-γ and sometimes IL-17A (38). NKTCs particularly secrete IL-4 or IFN-γ to inhibit or activate, respectively, the immune responses (38). MAITs are frequent, for example, in the liver where they represent up to half of the T cells (38). They particularly secrete IFN-γ and TNF-α in response to metabolites of the riboflavin synthesis of bacteria or fungi (e.g., *A. fumigatus*) (38, 163). Contributions of γδ T cells to the immune response are not fully understood (38). They are rare (i.e., < 5% of all T cells), mainly found in epithelia, and known to secrete pro-inflammatory cytokines (e.g., IL-17) (152). Tregs inhibit the function of other

T cells, B cells, and NKCs, to maintain balance between a healing and a self-damaging immune response (38, 50). They inhibit the immune response via IL-10 and TGF-β as well as via absorption of IL-2 by IL-2 receptors (38).

Soluble Interleukin 2 Receptor. Soluble interleukin 2 receptor (sIL-2R) antagonizes the effect of IL-2 by binding and thus neutralizing it (38). Tregs consistently produce high-affinity membrane receptors for interleukin 2 (IL-2R). If T cells are chronically or strongly stimulated, Tregs produce many IL-2Rs which then are shed from the cell surface, forming sIL-2R. sIL-2R equally binds IL-2 like IL-2R, also inhibiting the immune response (164). Higher sIL-2R levels in serum were associated with a higher risk for developing an IFI (92, 164). However, some malignant cells (e.g., as part of hematological malignancies) also intensively shed IL-2R, so sIL-2R was also associated with tumor activity (165).

Factors Influencing T Helper Cell Subset Differentiation. DCs are one factor influencing Th cell subset differentiation in draining lymph nodes (50). They can phagocytose spores and hyphae of *Aspergillus*, triggering T cell differentiation towards Th1s via IL-12 when phagocytizing spores, in contrast to triggering the differentiation to Th2s or Tregs via IL-4 and IL-10 when phagocytizing hyphae (50, 118). DCs can also drive the differentiation towards Th17s via IL-23 (in the presence of IL-1β and/or IL-6 and possibly TGF-β) after coming into contact with β-glucans from *Aspergillus* and *Rhizopus* hyphae (38, 119, 150). The various possible differentiations are a result of the activation of different receptors on DCs by the respective fungal morphologies, followed by the activation of different intracellular signaling pathways (50). Similar findings have been described for *Candida*: Phagocytosis of the yeast form by DCs is associated with initiation of Th1 differentiation, whereas the phagocytosed hyphal form promotes Th2 differentiation. However, the interaction between the fungus and the DC is not influenced just by the morphology of the fungus but also by the local tissue as well as opsonins attached to the surface of fungal cells.

In summary, IA elicits a mixed Th1- and Th2-like immune response, but while Th1-dominant responses are considered effective, Th2-dominant responses are associated with disease progression (8, 105, 166). Disease progression can be fueled by glucocorticoids which in general impair inflammatory reactions, T cell differentiation and proliferation, and leukocyte migration, while specifically dampening the Th1 response (114, 166). Similarly, mycotoxins (e.g., gliotoxin or citrinin from *A. fumigatus*) can also impair T cell proliferation

and vitality and stimulate a less protective Th2 response (114, 166). In IFIs, however, the role of a Th17 response is still debated (105). On the one hand, it triggers a protective pro-inflammatory, cellular-based immune response (38, 151, 167). On the other hand, there is evidence that the Th17 response is less protective than a Th1-dominant response (and possibly even suppressing a Th1 response) and rather harmed the host by causing an intense inflammatory reaction (153).

However, despite the observations that certain infections trigger specific Th cell responses that could be utilized in diagnostics, it must be noted that the concept of clearly defined Th cell subsets that secrete defined sets of cytokines is a substantial simplification of the immune system (38, 162, 168). First, the effect attributed to a cytokine may vary, depending on its concentration, the combination with other cytokines, or available target cells (162, 168). Next, individual Th cells often secrete cytokine combinations that diverge from the subset definitions (38, 162, 168). Then, different types of Th cell subsets are always present at the same time, cross-regulating each other while forming the overall immune response (93). In general, a specific Th cell subset shares only up to 5% of T cells in circulation; however, the actual proportions of subsets within the total amount of Th cells may substantially vary in the specific case (38, 97). Nevertheless, Th cells provide only part of the overall cytokine profile which is composed by the entirety of immunologically active cells (33, 38, 93). Therefore, in vitro findings on Th cell responses cannot be readily transferred to the actual clinical scenarios but require verification in clinical studies.

Diagnosis

A definite diagnosis of an IMI requires sterile tissue biopsies or sterile body fluids that demonstrate (a) hyphae in microscopy plus evidence of tissue damage or immune reactions, or (b) cultural growth of molds (16, 29, 127). The gold standard for the subsequent identification of the mold cultured is DNA amplification via polymerase chain reaction (PCR), followed by amplicon sequencing (127, 169). However, proving an IMI is challenging because cultures can take several weeks and often yield false-negative results, and invasive diagnostic procedures may be inappropriately risky as patients are often seriously ill (169, 170). In addition, there are often other concomitant non-fungal infections with similar clinical and radiological pictures, concealing the parallel fungal infection. Furthermore,

non-infectious pulmonary pathologies (e.g., radiation-induced pneumonitis, graft-versus-host disease [GVHD]) can mimic infectious diseases (1, 171, 172).

The diagnostic process starts with collecting medical history and a physical examination to assess pre-test probability and to identify risk factors for IMIs. Significant risk factors include neutropenia and an impairment of phagocytosis, of cytotoxic defense mechanisms, or of the T cell response that often occurs due to immunosuppressive medication (e.g., glucocorticoids or T cell suppressing medication; see Table 6 for details.) (6, 173, 174).

Three basic groups of patients can be identified who frequently accumulate enough individual immunosuppressive factors to be at high risk of developing an IMI:

- patients with hematological malignancies, especially when they receive intensive chemotherapy regimens or allogenic HCTs (9, 175, 176);
- solid organ transplant recipients, especially those who had lung, heart-lung, or liver transplantation, because they require a proportionally risky immunosuppressive medication over a long period of time to prevent transplant rejection (9, 21, 175);
- patients who had an IFI in the past when they again receive intensive chemotherapy regimens or immunosuppressive therapies, independently of the underlying disease (176).

Pre-existing alterations of the lung tissue (e.g., bronchiectasis, cavities, advanced chronic obstructive pulmonary disease) or acute severe viral pneumonia (e.g., due to influenza, severe acute respiratory syndrome coronavirus 2, or cytomegalovirus [CMV]) also entail a higher risk of coming down with IMIs, though the risk is not as high as with a severely impaired immune system. But due to the altered airway epithelium, spores find a better environment for germination and tissue invasion, and risk for developing an IMI particularly rises when patients with these pre-existing conditions receive high-dose glucocorticoid therapies (9, 40, 171).

In immunocompromised patients, the most frequent primary sites of IMIs are the lungs and the paranasal sinuses (7, 175, 177). Common symptoms in neutropenic patients with pulmonary IMIs include fever, dyspnea, cough, thoracic pain, and hemoptysis but are generally unspecific (1, 7, 9). Stridor may occur in *Aspergillus* tracheobronchitis (178). Nevertheless, symptoms often appear less severe in immunocompromised patients and these patients may even be oligo- or asymptomatic at the onset of IMIs (7, 8). Physical examination is not specific enough to discriminate between fungal and non-fungal infections (26, 33).

Table 6 *Risk Factors for Invasive Mold Infections*

Risk factor		References
	Neutropenia	
Severity	< 500–1000/μL neutrophils [a]	Donnelly et al. (127); Heinz et al. (179); Ruhnke et al. (176)
Duration	High risk if > 7–10 days [a]	Donnelly et al. (127); Heinz et al. (179); Ruhnke et al. (176)
Relevant period [b]	Immediately [a] preceding	Donnelly et al. (127)
	Glucocorticoids	
Dosage per day	Moderate dose or higher, e.g., ≥ 0.3 mg/kg body weight [a] prednisone equivalent	Chatham (55); Donnelly et al. (127)
Duration	Longer than short term, e.g., > 14–21 days [a]	Chatham (55); De Pauw et al. (125); Donnelly et al. (127)
Relevant period [b]	≤ 60 days [a]	Donnelly et al. (127)
	Immunosuppressants affecting T cell functions [c]	
Nucleoside analogues	e.g., purine analogues (e.g., azathioprine, mercaptopurine, fludarabine)	Donnelly et al. (127); Wingard (4)
Anti-thymocyte globulin		Wingard (4)
Cytokine specific mabs	e.g., anti-TNF-α (e.g., infliximab)	Donnelly et al. (127); Maus & Lionakis (180); Wingard (4).
Calcineurin inhibitors	e.g., cyclosporine, tacrolimus	De Pauw et al. (125); Donnelly et al. (127)
T cell specific mabs	e.g., anti-CD52 (e.g., alemtuzumab)	De Pauw et al. (125); Donnelly et al. (127); Wingard (4)
Janus kinase inhibitors	e.g., ruxolitinib, baricitinib	Maus & Lionakis (180)
Chimeric antigen receptor T cell therapy		Wingard (4); Maus & Lionakis (180)
Relevant period [b]	≤ 90 days [a]	Donnelly et al. (127)

Risk factor		References
Other Immunosuppressants [c]		
Bruton's tyrosine kinase inhibitor	e.g., ibrutinib	Donnelly et al. (127); Maus & Lionakis (180); Wingard (4).
Anti-C5a mab [d]	e.g., eculizumab	Maus & Lionakis (180)
Diseases with (chronic) impairment of the cellular immune response		
Congenital	e.g., chronic granulomatous disease, STAT3 deficiency	Donnelly et al. (127); Patterson (9)
Acquired	e.g., leukemias, lymphomas, acquired immunodeficiency syndrome	Fernández-Ruiz et al. (178); Finberg & Regierer (1); Patterson (9)
Special metabolic situations (only for mucormycosis)		
Diabetes mellitus	Particularly with ketoacidosis	Hospenthal & Walsh (174); Skiada et al. (21)
Iron overload	Particularly if treated with deferoxamine	Hospenthal & Walsh (174); Skiada et al. (21)

Note. CD = cluster of differentiation; mab = monoclonal antibody; STAT3 = signal transducer and activator of transcription 3; TNF = tumor necrosis factor.

[a] The detailed specifications regarding neutropenia, glucocorticoids, and the relevant time periods are to be understood as guidance for risk estimation, rather than as rigid cut-off values as the underlying consensus definitions of the European Organization for Research and Treatment of Cancer/Infectious Diseases Group/Mycoses Study Group Education and Research Consortium (127) are intendedly conservative with strict thresholds to achieve better accuracy in diagnostic studies but are not intended to be utilized for disease definition in clinical routine. [b] Indicates the relevant period preceding the onset of clinical features (e.g., symptoms, radiologic findings) that are suspicious of an invasive mold infection. [c] Due to the high and continuously growing number of medications that can impair the cellular immune response, only a selection of frequently cited substances and substance classes is presented. [d] Only in combination with other risk factors.

Cellulitis, abscesses, ulcers, or necroses of the skin or mucosa are the most helpful findings in physical examination, but in these cases, IMIs have commonly already progressed to dissemination (15, 26).

Computed Tomography of the Chest

Thin-section computed tomography (CT) is the method of choice for radiologic evaluation of pneumonias in febrile neutropenic patients at risk for IFIs (1, 176, 181). The most specific radiological signs of IMIs were exemplary listed in the clinical criteria of the 2008 revised European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC)/ National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) definitions, published by De Pauw et al. (125): “Dense, well-circumscribed lesions(s) with or without a halo sign” (p. 1817, Table 2), air-crescent signs, and cavities. In the 2020 revision, published by Donnelly et al. (127), “Wedge-shaped and segmental or lobar consolidation” (p. 1370, Table 2) were added as a relevant radiologic finding, as well as the reverse halo sign that can indicate IMIs other than IPAs and is particularly known for its occurrence in mucormycosis. Noduli and pleural-based wedge-shaped lesions, unilaterally as well as bilaterally, are common findings especially in early IMIs, but larger consolidation may also occur early due to infarctions (8, 182, 183). In IPAs, halo signs are particularly found in the first week but are less frequent after 2 weeks anymore (182, 184, 185). Similarly, the reversed halo signs (synonym: atoll sign) are more frequent in the first week of pulmonary mucormycosis but are usually not observed anymore after 2 weeks (186-188). The lesions can coalesce to larger masses over time (183). Within the first week, the lesions usually increase in volume even despite adequate antifungal therapy (184). Over time, nodules with halo signs or reversed halo signs commonly evolve into air-crescent signs or cavities (186, 189). In fact, air-crescent signs characterize the later radiological stage of an IPA and are rare in early stages but visible in almost two out of three cases after 2 weeks, usually following recovery from neutropenia (8, 182, 184). However, these are no really specific radiologic patterns for IMIs. For example, the halo sign is most times caused by IPAs in immunocompromised patients but can generally occur in any angio-invasive IFI, as well as in other infectious or non-infectious inflammatory processes or in neoplastic diseases (185, 190). Particularly from approximately 7 to 14 days after the first noduli have arisen, chest CT scans mostly feature just unspecific signs (184).

In general, pulmonary IMIs resemble each other radiologically due to their likewise capability to invade blood vessels, making it difficult to distinguish IMIs from each other by radiologic evaluation (29, 191-194). Comparing mucormycosis and IPA, however, 10 or more nodules, small bilateral pleural effusions, abscesses, or reversed halo signs are more characteristic of mucormycosis than of IPA (183, 194, 195). Particularly pleural effusions are uncommon in IPAs, occurring only in up to 10% of cases (7, 183, 195).

Microscopy and Culture

Microscopy or culture of sterile material remain the diagnostic gold standard for proving IMIs, despite recent advances with less invasive methods (1, 176). However, sterile tissue biopsies or body fluids require invasive procedures, which can be risky, and subsequent microscopy and culture have limited diagnostic performance (169, 170, 196). Consequently, the cost-benefit ratio of proving IMIs by invasive procedures is often limited in clinical practice. Nevertheless, microscopic evaluation is relatively fast, though distinguishing molds solely by microscopic morphology is difficult, as genera often differ only slightly in hyphal septation and branching patterns (175, 196, 197). For some common mold genera, however, immunohistochemical staining is available that increases visibility of hyphae and sensitivity of histopathological evaluations (198). Nevertheless, the specificity of the antibodies remains unsatisfactory, and molecular techniques (e.g., PCR, fluorescence in situ hybridization [FISH]) are superior to immunohistochemical identification (188, 196). Contamination of sterile samples can cause false-positive microscopy results in principle but this is rare (196). False-negative results can occur with small fungal burden if, for example, infarcted necrotic tissue is unintentionally obtained by biopsy instead of tissue infiltrated with hyphae (7, 199). In addition, antifungal therapies also reduce sensitivity (199). For severely cytopenic patients, obtaining BALF is less risky than biopsies or needle aspirations. However, the sensitivity of microscopic evaluation of BALF is limited (~50%), and as BALF is not sterile, the specificity is also low (200, 201).

Cultures of tissue biopsies can only identify 43%–63% of causative molds, even when hyphae are microscopically visible (202, 203). Hyphae of Mucorales are particularly vulnerable to preceding tissue preparations (15, 16). The presence of mold-active antifungals in the sample material additionally lowers sensitivity of cultures (13). Blood cultures are relatively sensitive to disseminating fusariosis (40%–70%) or lomentosporiasis (up to 80%) but with most other IMIs, particularly in IA and mucormycosis, blood cultures lack sensitivity

(37, 204-208). Published performance characteristics for cultures with respiratory samples to diagnose IMIs are heterogeneous; this is at least partly caused by varying inclusion criteria, inconsistencies in case classifications, variations in study sizes, different timing of sample collection within the course of disease, and incorporation bias (199, 209). Some authors considered the sensitivity of cultures from respiratory and tissue samples to diagnose IPAs to be roughly 10%–30% (7). However, reports of 50%–60% sensitivity for cultures with BALF and/or sputum also exist (201, 209). Importantly, cultures with BALF are significantly superior to cultures with sputum as cultures with sputum become positive only late in the course of IMIs and are generally less sensitive and specific than cultures with BALF (2, 199). Administering mold-active antifungals before sampling additionally impacts the sensitivity of respiratory samples (13, 210). Moreover, cultures lack sensitivity for mixed fungal infections, particularly in case of an accompanying non-*Aspergillus* mold (13, 210). Colonization of the host and contamination of samples or sample containers commonly cause false-positive results with non-sterile samples (6, 7, 211). Thus, the relevance of a mold growing from respiratory samples depends on the underlying risk factors for an IMI (8, 212). Most mold-positive respiratory cultures are indeed associated with an actual IMI when the host is at high risk and symptoms or radiological findings are coherent with an IMI (13, 192, 213). Contrary, this association is significantly smaller in constellations without a high risk (212).

Enzyme-Linked Immunosorbent Assays for Galactomannan

Cell walls of *Aspergillus* contain the polysaccharide antigen galactomannan which is released during hyphal growth, and it was demonstrated that its concentration in serum correlates with fungal burden (45, 214). A widely utilized commercial assay for galactomannan is the Platelia assay (213). Its monoclonal antibody EB-A2 detects the β-1,5-galactofuranose side chain of galactomannan when at least four residues long (213, 215). Initially, it was assumed that this assay was entirely specific for *Aspergillus* among clinically relevant fungi, but reactions with galactofuranose-containing molecules from other sources are possible, and various cross-reactions with other fungi were reported after clinical implementation (see Table 7) (213, 214). In fact, due to common cross-reactivity, the usefulness of EB-A2-based assays for diagnosing invasive fusariosis caused by species of the *Fusarium solani* or *F. oxysporum* complexes is discussed (216). Another disadvantage is that galactomannan assays are not available in every laboratory.

Table 7 *Non-*Aspergillus *IFIs Reported to Cause False-Positive Galactomannan Results*

Fungus	Reference
Cryptococcus spp.	Huang et al. (217); Xavier et al. (218).
Fusarium spp.	Ruhnke et al. (176).
Histoplasma capsulatum	Ruhnke et al. (176); Xavier et al. (218).
Paracoccidioides brasiliensis	Xavier et al. (218).
Penicillium spp. [a]	Cummings et al. (219); Swanink et al. (220).
Purpureocillium lilacenum [a, b]	Cummings et al. (219).
Rasamsonia argillacea [c]	Valentin et al. (221).
Talaromyces marneffei [d]	Huang et al. (217).
Trichosporon dermatis	Fekkar et al. (222).

Note. The sources cited in this table include guidelines, case reports, and in vitro experiments. IFI = invasive fungal infection.

[a] Demonstrated in vitro. [b] Formerly *Paecilomyces lilacinus* (223). [c] Formerly *Penicillum argillaceum* and *Geosmithia argillacea* (223). [d] Formerly *Penicillium marneffei* (223).

Furthermore, those laboratories that do offer the test often delay sample analysis because of economic reasons, until an adequate number of individual samples have been collected (224). Sometimes this delays the results for several days, and by this way early treatment initiation is hampered, and improvement of the patients' outcomes is failed (176, 225).

In their meta-analysis, Leeflang et al. (226) calculated 78% sensitivity, 95% confidence interval (CI) [70%, 85%], and 85% specificity, 95% CI [78%, 91%], for diagnosing IA with galactomannan in serum at a cut-off of 0.5 optical density index (ODI). However, many underlying studies were based on a multiple measurement setting which overestimates the performance of an individual measurement. Rising the cut-off to 1.0 ODI increased accuracy via higher specificity (90%, 95% CI [86%, 93%]) but at the expense of sensitivity (71%, 95% CI [63%, 78%]) (170, 226). Baseline concentrations of galactomannan as well as kinetics in serum correlated with therapy response and survival; thus, the assay provided an additional prognostic value (214, 227). However, specific threshold values for prognostic purposes have not been established yet (214). Regarding false-positive results with serum, the half-life of cross-reacting substances, for example received via infusions, is about 2–3 days (214, 228). Thus, it may take several days for galactomannan assays to produce

true-negative results again after such an incident. However, necessary time for elimination also depends on concomitant impairments of the elimination pathways (i.e., liver, kidney, neutrophils) (214, 228). False-negative results occur if angioinvasion is limited or the passage of galactomannan into the bloodstream is restrained or too low in relation to its elimination rate (45, 214, 225, 229, 230). Important known causes of false-positive (apart from cross-reactions with other fungi, see Table 7 for those) and false-negative results are listed in Table 8.

As BALF is collected at the site of infection, galactomannan is often detected earlier in BALF than in serum (33). In addition, the diagnostic accuracy of galactomannan assays is better with BALF than with serum (231). In a meta-analysis, de Heer et al. (231) calculated 88% sensitivity, 95% CI [75%, 100%], and 81% specificity, 95% CI [71%, 91%], with a cut-off of 0.5 ODI. A cut-off of 1.0 ODI provided 78% sensitivity, 95% CI [61%, 95%], and 93% specificity, 95% CI [87%, 98%]. While the difference in sensitivity of both cut-offs was not statistically significant, the difference in specificity was. Another meta-analysis by Zou et al. (232) with a higher number of included studies calculated 87% or 86% sensitivity and 89% or 95% specificity for a cut-off of 0.5 ODI or 1.0 ODI, respectively. However, the study inclusion criteria by Zou et al. were less restrictive regarding methodological quality than the inclusion criteria by de Heer et al., and even not a single study included by de Heer et al. met all assessment criteria for low risk of bias (231, 232). The causes of false results with BALF are comparable to those affecting serum samples. Additional factors include a possible colonization of the airways with *Aspergillus spp.* and the virtually unavoidable variabilities when performing a bronchoalveolar lavage (BAL) (231, 233, 234). Although not officially validated for clinical practice, galactomannan concentrations can also be determined in tissue biopsies, cerebrospinal fluid, pleural fluid, sputum, and urine (203, 235, 236). For clinical trials, however, only cerebrospinal fluid is accepted as an additional sample type apart from plasma, serum, and BALF (127).

Coagulation Cascade Activation by 1,3-ß-D-Glucan

Polysaccharides containing BDG trigger the coagulation cascade of horseshoe crabs via factor G activation (213, 237, 238). The end products of this cascade can be measured and allow to conclude the BDG concentration in the sample (237, 239). BDG is a cell wall component of many fungi; Table 9 shows fungal genera with clinically relevant species that commonly release enough BDG to cause elevated levels in serum.

Table 8 *Other Causes of False Results With Galactomannan Assays*

Known causes	Comment	References
	False-positive results	
β-Lactam antibiotics	Reported for piperacillin-tazobactam, sulbactam-ampicillin, and amoxicillin-clavulanate	Aubry et al. (228); Mercier et al. (214); Metan et al. (240)
	Less frequent nowadays; contaminating production processes should have been adapted.	Mikulska et al. (241); Ruhnke et al. (176)
Gluconate-containing electrolyte solutions	Reported for solutions for direct use (e.g., as an infusion or lavage fluid for BALs) or for the preparation of infusion solutions.	Martín-Rabadán et al. (242); Mercier et al. (214); Patterson (213)
Blood products	Reported for erythrocyte and platelet concentrates, FFP, Igs, among others	Martín-Rabadán et al. (242); Ramsay et al. (243)
Food	Uptake of cross-reacting substances during gastrointestinal mucositis (e.g., due to chemotherapy, radiation, severe GVHD)	Denning (244); Guigue et al. (245)
Colonization of airways with *Aspergillus spp.*	Affects BALF	de Heer et al. (231); Patterson (213)
	False-negative results	
Encapsulated infectious processes (e.g., abscesses)	Insufficient amount of GM passes into the bloodstream.	Verweij et al. (230).

Known causes	Comment	References
Effective mold-active therapy for 2 days or longer	Limited angioinvasion (affects serum) and fungal growth (affects serum and BALF)	Marr et al. (229); Mennink-Kersten et al. (45); Racil et al. (234); Reinwald et al. (246).
	Performance of GM should be preserved with ineffective prophylaxis/therapy, as seen in breakthrough/progressing IA.	Hoenigl et al. (247); Vena et al. (248).
Non-neutropenic state	Limited angioinvasion; smaller fungal burden; GM elimination via neutrophils	Mercier et al. (214).
Dilution effect in BALF	Standardization of BAL is challenging.	Hsu et al. (233); Racil et al. (234)

Note. A list of fungal genera other than *Aspergillus* that also cause false-positive results due to cross-reactions is provided in Table 7. BAL = bronchoalveolar lavage; BALF = bronchoalveolar lavage fluid; FFP = fresh frozen plasma; GM = galactomannan; GVHD = graft-versus-host disease; Ig = immunglobulin.

Mucorales release only little to no BDG (26, 188, 249). Moreover, the diagnostic value of BDG for certain IMIs remains unclear. For example, *Scedosporium*, *Lichtheimia*, and *Cunninghamella* do contain BDG, but it is only released in very low amounts, and a large fungal burden may be required for reaching the cut-off value of the assays (249-251). BDG in serum can precede other evidence of IFIs a few days ahead, but levels may fluctuate over time and are not reliably linked to the outcome (252).

A meta-analysis by White et al. (253) revealed 83% sensitivity, 95% CI [74%, 89%], and 79% specificity, 95% CI [64%, 88%]. When the authors applied more strict inclusion criteria for studies to minimize spectrum bias, however, the specificity decreased noticeably to 63%, 95% CI [~35%, ~85%], while the sensitivity remained similar with 80%, 95% CI [~60%, ~90%] (253, Figure 5). Moreover, these performance metrics refer to BDG-producing IFIs in general but cannot be applied to diagnose IMIs without restriction (253).

Table 9 *Clinically Relevant Fungi Sufficiently Secreting BDG*

Genus	Reference
Molds	
Aspergillus	Angebault et al. (251); Odabasi et al. (237); Pickering et al. (254)
Fusarium	Angebault et al. (251); Odabasi et al. (237)
Non-molds	
Candida	Angebault et al. (251); Odabasi et al. (237); Pickering et al. (254)
Coccidioides	Al-Obaidi et al. (255); Angebault et al. (251)
Histoplasma	Angebault et al. (251); Pickering et al. (254)
Pneumocystis	Karageorgopoulos et al. (256); Son et al. (257)
Trichosporon	Angebault et al. (251); Odabasi et al. (237)

Note. These are fungal genera that were regularly observed in conjunction with elevated serum levels of 1,3-β-D-glucan (BDG). This list is not exhaustive; there are other, less frequently observed associations between invasive fungal infections and positive BDG assays that are not included in this list. For example, see Angebault et al. (251) for more information.

In addition, there are many other causes of false test results, especially of false-positive results (see Table 10). Thus, BDG has limited specificity, and its usefulness in diagnosing IMIs is doubted (127, 170). As a result, BDG was removed from the mycological criteria defining IMIs of the 2020 EORTC-IDG/MSGERC consensus definitions (127).

Polymerase Chain Reactions With Clinical Samples

PCR has become an important diagnostic tool for IMIs (7, 181). DNA amplification via PCR and subsequent sequencing of the amplicon is the gold standard for the exact identification of cultivated fungi (127, 169). Additionally, PCR performed with material from formalin-fixed and paraffin-embedded sterile tissue samples with visible hyphae was accepted by the EORTC-IDG/MSGERC in 2020 for identifying the causative genus (127). As for the detection of molds in clinical samples, the *Aspergillus* PCR is the best investigated to date (127). Sufficient progress has been made in its standardization, and good-quality *Aspergillus* PCR assays are now commercially available.

In addition, a semi-quantitative multiplex real-time PCR targeting Mucorales in clinical samples is now commercially available (i.e., MucorGenius, covering *Rhizopus*, *Mucor*, *Lichtheimia*, *Cunninghamella*, and *Rhizomucor*) (258, 259).

Table 10 *Causes of False Results With BDG Assays*

Known causes	Comment	References
	False-positive results	
Dialysis	If cellulose membranes are used [a]	Kanda et al. (260); Kato et al. (261); Koo et al. (262); Marty & Koo (263)
Intravenously applied products	Intravenous drug application in general: Contamination via cellulose membranes used as filters in infusion sets. Blood-derived products (e.g., Igs, coagulation factors, albumin): Contamination via cellulose membranes during production. Antimicrobial drugs (e.g., amoxicillin-clavulanic acid): Contamination via cellulose membranes, use of fungi in the production process, or cross-reactions. Parenteral nutritional solutions containing β-glucans or cross-reactive substances.	Koo et al. (262); Marty & Koo (263); Mennink-Kersten et al. (264); Ruhnke et al. (176)
Gauzes	If β-glucan containing gauzes have contact to serosal membranes during/after surgery	Kimura et al. (265); Marty & Koo (263)
Hemolytic samples	Interference with assay	Pickering et al. (254)
Bacteremia (e.g., due to *Pseudomonas aeruginosa* or *Streptococcus pneumoniae*)	Release of β-glucans by bacteria (controversially discussed)	Digby et al. (266); Marty & Koo (263); Mennink-Kersten & Verweij (267)

Known causes	Comment	References
Mucositis enterocolitis	Gastrointestinal uptake after oral intake of β-glucans (controversially discussed)	Ellis et al. (252)
	False-negative results	
Intravenous antimicrobial drugs (e.g., pentamidine)	Inhibition of assay	Marty et al. (268); Marty & Koo (263)
High concentrations of bilirubin or triglycerides	Inhibition of assay	Pickering et al. (254)

Note. Ig = immunoglobulin.

[a] Particularly unmodified membranes have been identified to be causative; however, some modified cellulose membranes have also been observed to be affected (260, 261).

However, no PCR assays for *Fusarium*, *Scedosporium*, or *Lomentospora* have been made commercially available for clinical samples yet, but in-house assays for *Fusarium* have been published and evaluated by Dellière et al. (269), and assays for *Scedosporium* and *Lomentospora* by Harun et al. (270), for example. Pan-fungal PCR approaches help to quickly identify rare or refractory IFIs (11). In-house pan-fungal PCR assays were published by Zeller et al. (271), Boch et al. (272), and Lau et al. (273), for example. Common causes of false PCR results are listed in Table 11.

A meta-analysis of the *Aspergillus* PCR with blood or serum demonstrated 79% sensitivity, 95% CI [71%, 86%], and 80% specificity, 95% CI [70%, 87%] (274). The MucorGenius assay retrospectively produced 75% sensitivity, 95% CI [48%, 93%]; evaluation included whole blood, serum, and plasma samples (259).

Another meta-analysis evaluated the *Aspergillus* PCR performed with BALF and demonstrated 90% sensitivity, 95% CI [77%, 96%], and 96% specificity, 95% CI [93%, 98%] (but possible IPAs were excluded) (275). The MucorGenius assay achieved similar values for diagnosing pulmonary mucormycosis with BALF (i.e., 90% sensitivity and 98% specificity); however, results from tracheal aspirates, sputum samples, pleural fluid, and lung biopsies were also included in that analysis, and possible IMIs were excluded (258).

Table 11 *Causes of False Results With PCR Assays for Molds*

Kown causes	Comment	References
	False-positive results	
Colonization	Affecting BALF/non-sterile sample sites	Chen et al. (276); Zeller et al. (271)
Contamination	Various error sources, including contamination of reagents or sample containers	White, Bretagne, et al. (277); Zeller et al. (271)
	False-negative results	
Suboptimal DNA extraction	Meet standardization recommendations of the FPCRI	Mennink-Kersten et al. (278); White, Bretagne, et al. (277); White, Perry, et al. (279); White, Mengoli, et al. (280)
Effective mold-active antifungal prophylaxis/therapy	Due to the smaller fungal burden and less invasiveness	Avni et al. (275); Racil et al. (234); Reinwald et al. (281)
Inhibition of assays		Chen et al. (276); Lass-Flörl et al. (282); White, Bretagne, et al. (277)
Mixed fungal infection	Affects multiplex assays	Lass-Flörl et al. (282); Zeller et al. (271)
Dilution effect in BALF	Standardization of BAL is challenging.	Racil et al. (234)
Sample preparation	Particularly affecting formalin-fixed tissue samples	Gholinejad-Ghadi et al. (283); Zeller et al. (271); Lau et al. (273)

Note. This table provides an overview of the most common causes of false results when using polymerase chain reaction (PCR) assays for the diagnosis of invasive mold diseases.
BAL = bronchoalveolar lavage; BALF = bronchoalveolar lavage fluid;
DNA = deoxyribonucleic acid; FPCRI = fungal polymerase chain reaction initiative;
PCR = polymerase chain reaction.

With non-fixed samples, PCR assays achieved 96%–100% sensitivity and 86%–93% specificity (202, 282, 283). Gohlinejad-Ghadi et al. (283) demonstrated a notable decrease in sensitivity from 100% to 56% for a Mucorales PCR assay when samples were fixed with formalin and embedded in paraffin instead of utilizing non-fixed samples. This is because formalin fixation partially destroys DNA in the samples (188).

Immunochromatographic Lateral-Flow Devices

Lateral-flow devices (LFD) are promising options for diagnosing IAs. Their major advantages are user-friendliness and rapid turnaround times. One available LFD, the *Aspergillus* Lateral-Flow Device (AspLFD), is based on the monoclonal antibody JF5, which reacts with an extracellular mannoprotein antigen that is secreted during active growth of *Aspergillus* hyphae (213, 284, 285). Apart from centrifugation, pre-treatment of BALF is not necessary, therefore results can be available shortly after or even during bronchoscopy (285). In contrast, heating is necessary with serum, but this is still easy and quick to perform in laboratories, and substantially faster than an enzyme-linked immunosorbent assay (ELISA), an enzyme cascade assay, or a PCR (224, 286). To diagnose IA, Pan et al. (284) calculated a sensitivity of 68%, 95% CI [52%, 81%], and a specificity of 81%, 95% CI [75%, 87%], for the AspLFD when used with serum. When performed with BALF, the overall sensitivity was 86%, 95% CI [76%, 93%], and 84% specificity, 95% CI [80%, 88%]. Subsequently to Pan et al., our research group published a review regarding the performance of the AspLFD with BALF (287). We concluded a lower overall sensitivity of 73% and a specificity of 90% to diagnose IPAs (possible IPAs were excluded in analysis) (284, 287).

Meanwhile, the IMMY sona *Aspergillus* Galactomannan Lateral Flow Assay (AGM-LFA) was developed, but it requires a slightly larger effort in sample preparation than the AspLFD as not only serum but also BALF must be pretreated (288-290). However, the AGM-LFA still requires significantly less workload, equipment, and time than an ELISA. With serum samples, the AGM-LFA reached 79% sensitivity and 81% specificity for diagnosing IA (possible IAs were excluded); additionally, the results of the AGM-LFA (read out with a digital device) and the Platelia galactomannan ELISA (using a cut-off of 0.5 ODI) have been demonstrated to agree with each other, $\kappa = 0.61$ (288). With BALF, the AGM-LFA had a sensitivity of 89% and a specificity of 88% for detecting IPA (possible IPAs and some cases without IPA were excluded) and was similar specific but more sensitive in direct comparison with the AspLFD (289).

Study Objectives

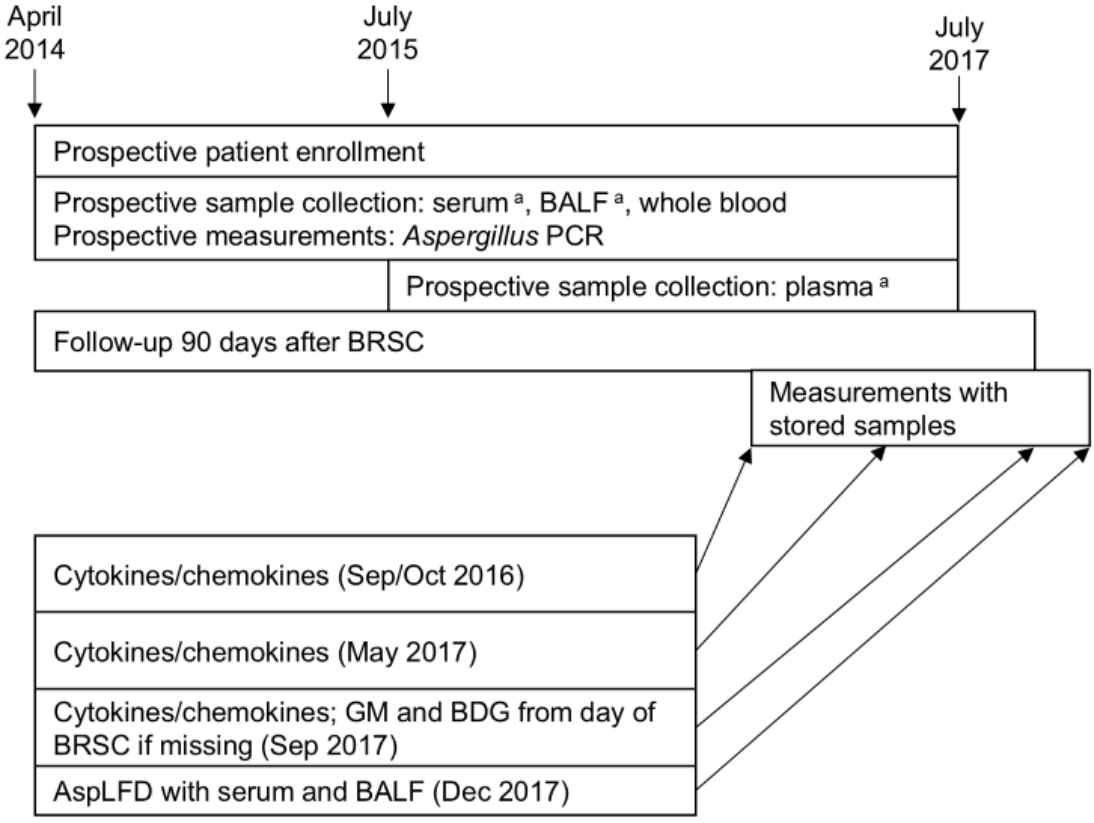
The primary aim of this study was to identify new immunological biomarkers in serum and BALF for diagnosing IMIs. The target population were patients with underlying hematological malignancies, suspected pulmonary infections, and receiving mold-active antifungals. The following biomarkers were selected after a literature search whose results have been summarized in the preceding parts of the introduction: IFN-γ, IL-4, IL-6, IL-10, IL-15, IL-17A, IL-22, TNF-α, sIL-2R, IL-8, and CCL5. The performance of these biomarkers to diagnose an IMI should be calculated and compared to other diagnostics, namely, microbiological (culture with blood and BALF), antigen-based (galactomannan and AspLFD with serum and BALF, BDG with serum), and molecular (*Aspergillus* PCR with ethylenediaminetetraacetic acid [EDTA] blood and BALF) methods. Secondary objectives included

- determining antifungal therapies at the time of sampling to estimate their impact on diagnostics and the conceivable influence of biomarkers on treatment decisions;
- longitudinal evaluation of changes in concentrations of the biomarkers;
- and assessing the prognostic values of the biomarkers regarding overall 90-day mortality.

Material and Methods

The present study was designed as an observational cohort study, with patient recruitment and sample collection conducted prospectively. Patients were recruited at the Department of Internal Medicine, University Hospital Graz, Graz, Austria, from April 2014 to July 2017. Informed consent was obtained within 24 hours of bronchoscopy. Except for whole blood samples collected for PCR, samples were frozen and stored until collective measurements were performed on several days throughout the study period (see Figure 4). The follow-up period for mortality assessment spanned 90 days, commencing on the day of bronchoscopy.

Figure 4 *Organizational Procedures of the Study*



Note. Patient recruitment and sample collection were carried out prospectively; however, measurements were performed subsequently (except for *Aspergillus* PCR), using frozenly stored samples. AspLFD = *Aspergillus* Lateral-Flow Device; BALF = bronchoalveolar lavage fluid; BDG = 1,3-β-D-glucan; BRSC = bronchoscopy; GM = galactomannan; PCR = polymerase chain reaction.

[a] These sample types were stored frozenly for subsequent collective measurements.

Enrollment of Patients

The patients' attending physicians decided about the implementation of bronchoscopies. Specialists in pulmonology performed bronchoscopies at the endoscopy unit of the Department of Internal Medicine on a twice-weekly basis, on Mondays and Wednesdays. If necessary, additional bronchoscopies were performed at the intensive care unit or the bone marrow transplantation unit on other weekdays as well. The study investigators reviewed the bronchoscopy appointments and the medical history of the respective patients to evaluate if the inclusion criteria were met. It was hypothesized that approximately 85% of eligible patients would sign informed consent, and enrollment of at least 100 participants was intended. The following inclusion criteria had to be met by the patient to participate:

- presence of a hematological malignancy as underlying disease
- pulmonary infection was suspected
- bronchoscopy with BALF sampling was done
- age was 18 years or older
- informed consent was provided.

The local ethics committee of the Medical University of Graz provided its approval for the study (EC-numbers 25–221 and 23–343). The study was consistent with the Declaration of Helsinki (291) and Good Clinical Practice standards, and was registered on ClinicalTrials.Gov (identifiers: NCT02058316 and NCT01576653).

Cases were excluded for two reasons: (a) BALF or serum samples collected on the day of bronchoscopy were not available, or (b) inclusion criteria were apparently met initially but not confirmed in the following diagnostic process (e.g., suspected hematological malignancy that was not confirmed subsequently).

A comprehensive array of data was retrieved from the patient charts maintained at the ward during patient enrollment, as well as from the electronic data processing system of the Steiermärkische Krankenanstaltengesellschaft (Styrian Hospital Association; KAGes) after the enrollment. This included information regarding underlying diseases; previous therapies, including HCTs or antifungal medications; laboratory results including microbiological, molecular biological, and serological results regarding other infectious agents; radiological reports; histopathological and autopsy reports; and follow-up visits at outpatient clinics. The electronic data processing system of KAGes provided access to patients' data recorded by any

hospital of the KAGes in Styria, and, in some cases, to documentation of outpatient palliative care assistance institutions.

Endpoints

The primary endpoints of the study were the statistical characteristics of diagnostic accuracy on the day of bronchoscopy, namely, sensitivities, specificities, likelihood ratios (LR), and diagnostic odds ratios (DOR) of the 11 cytokines/chemokines under evaluation, of the *Aspergillus* PCR, and of the AspLFD. The reference for defining IMIs was the 2008 EORTC/MSG consensus definitions (125), comprising host criteria, clinical/radiological criteria, and mycological evidence. The secondary endpoints were as follows:

- presence of mold-active antifungal therapy
- difference in cytokine/chemokine levels before and after bronchoscopy
- all-cause mortality at 90 days following bronchoscopy.

Consensus Definitions as Reference Standard

The study cases were divided into four groups with different probabilities of IMI based on the 2008 EORTC/MSG consensus definitions (125): unlikely, possible, probable, and proven IMI. For cases to be categorized as proven IMI, a sterile sample that provided microscopic or cultural evidence of a mold was required (see Table 12 for details) (125).

For the categorization as a possible IMI, at least one host factor and one clinical criterion were required (see Table 13 for details) (125).

The criteria for probable IMI were met if, in addition to the criteria for possible IMI, there was evidence of a mold (a) by cytology, microscopy, or culture from non-sterile materials (i.e., BALF, bronchial brush, sputum, or sinus aspirates); (b) by galactomannan detection from serum or BALF; or (c) by BDG detection from serum (125). Differing from the 2008 EORTC/MSG definitions, we applied the approach that necessitated two consecutive positive galactomannan tests with serum before conceding the serum assessment as fulfilling the mycological criterion (226, 292). This variant of interpretation enhances the accuracy of screening by lowering frequency of false-positive galactomannan results findings without compromising sensitivity (226).

Table 12 *Criteria for Proven Invasive Fungal Disease Except for Endemic Mycoses*

Analysis and specimen	Molds[a]	Yeasts[a]
Microscopic analysis: sterile material	Histopathologic, cytopathologic, or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy in which hyphae or melanized yeast-like forms are seen accompanied by evidence of associated tissue damage	Histopathologic, cytopathologic, or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy from a normally sterile site (other than mucous membranes) showing yeast cells—for example, *Cryptococcus* species indicated by encapsulated budding yeasts or *Candida* species showing pseudohyphae or true hyphae[c]
Culture		
Sterile material	Recovery of a mold or "black yeast" by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding bronchoalveolar lavage fluid, a cranial sinus cavity specimen, and urine	Recovery of a yeast by culture of a sample obtained by a sterile procedure (including a freshly placed [<24 h ago] drain) from a normally sterile site showing a clinical or radiological abnormality consistent with an infectious disease process
Blood	Blood culture that yields a mold[d] (e.g., *Fusarium* species) in the context of a compatible infectious disease process	Blood culture that yields yeast (e.g., *Cryptococcus* or *Candida* species) or yeast-like fungi (e.g., *Trichosporon* species)
Serological analysis: CSF	Not applicable	Cryptococcal antigen in CSF indicates disseminated cryptococcosis

[a] If culture is available, append the identification at the genus or species level from the culture results.
[b] Tissue and cells submitted for histopathologic or cytopathologic studies should be stained by Grocott-Gomorri methenamine silver stain or by periodic acid Schiff stain, to facilitate inspection of fungal structures. Whenever possible, wet mounts of specimens from foci related to invasive fungal disease should be stained with a fluorescent dye (e.g., calcofluor or blankophor).
[c] *Candida*, *Trichosporon*, and yeast-like *Geotrichum* species and *Blastoschizomyces capitatus* may also form pseudohyphae or true hyphae.
[d] Recovery of *Aspergillus* species from blood cultures invariably represents contamination.

Note. From "Revised Definitions of Invasive Fungal Disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group," by B. De Pauw, T. J. Walsh, J. P. Donnelly, D. A. Stevens, J. E. Edwards, T. Calandra, P. G. Pappas, J. Maertens, O. Lortholary, C. A. Kauffman, D. W. Denning, T. F. Patterson, G. Maschmeyer, J. Bille, W. E. Dismukes, R. Herbrecht, W. W. Hope, C. C. Kibbler, B. J. Kullberg, K. A. Marr, P. Muñoz, F. C. Odds, J. R. Perfect, A. Restrepo, M. Ruhnke, B. H. Segal, J. D. Sobel, T. C. Sorrell, C. Viscoli, J. R. Wingard, T. Zaoutis, and J. E. Bennett (125), 2008, *Clinical Infectious Diseases*, *46*(12), p. 1817 (DOI: 10.1086/588660). Copyright 2008 by Infectious Diseases Society of America. Reprinted with permission.

Table 13 *Criteria for Probable Invasive Fungal Disease Except for Endemic Mycoses*

Host factors[a]
Recent history of neutropenia (<0.5 × 10^9 neutrophils/L [<500 neutrophils/mm^3] for >10 days) temporally related to the onset of fungal disease
Receipt of an allogeneic stem cell transplant
Prolonged use of corticosteroids (excluding among patients with allergic bronchopulmonary aspergillosis) at a mean minimum dose of 0.3 mg/kg/day of prednisone equivalent for >3 weeks
Treatment with other recognized T cell immunosuppressants, such as cyclosporine, TNF-α blockers, specific monoclonal antibodies (such as alemtuzumab), or nucleoside analogues during the past 90 days
Inherited severe immunodeficiency (such as chronic granulomatous disease or severe combined immunodeficiency)
Clinical criteria[b]
Lower respiratory tract fungal disease[c]
The presence of 1 of the following 3 signs on CT:
Dense, well-circumscribed lesions(s) with or without a halo sign
Air-crescent sign
Cavity
Tracheobronchitis
Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis
Sinonasal infection
Imaging showing sinusitis plus at least 1 of the following 3 signs:
Acute localized pain (including pain radiating to the eye)
Nasal ulcer with black eschar
Extension from the paranasal sinus across bony barriers, including into the orbit
CNS infection
1 of the following 2 signs:
Focal lesions on imaging
Meningeal enhancement on MRI or CT
Disseminated candidiasis[d]
At least 1 of the following 2 entities after an episode of candidemia within the previous 2 weeks:
Small, target-like abscesses (bull's-eye lesions) in liver or spleen
Progressive retinal exudates on ophthalmologic examination
Mycological criteria
Direct test (cytology, direct microscopy, or culture)
Mold in sputum, bronchoalveolar lavage fluid, bronchial brush, or sinus aspirate samples, indicated by 1 of the following:
Presence of fungal elements indicating a mold
Recovery by culture of a mold (e.g., *Aspergillus, Fusarium,* Zygomycetes, or *Scedosporium* species)
Indirect tests (detection of antigen or cell-wall constituents)[e]
Aspergillosis
Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF
Invasive fungal disease other than cryptococcosis and zygomycoses
β-D-glucan detected in serum

NOTE. Probable IFD requires the presence of a host factor, a clinical criterion, and a mycological criterion. Cases that meet the criteria for a host factor and a clinical criterion but for which mycological criteria are absent are considered possible IFD.

[a] Host factors are not synonymous with risk factors and are characteristics by which individuals predisposed to invasive fungal diseases can be recognized. They are intended primarily to apply to patients given treatment for malignant disease and to recipients of allogeneic hematopoietic stem cell and solid-organ transplants. These host factors are also applicable to patients who receive corticosteroids and other T cell suppressants as well as to patients with primary immunodeficiencies.

[b] Must be consistent with the mycological findings, if any, and must be temporally related to current episode.

[c] Every reasonable attempt should be made to exclude an alternative etiology.

[d] The presence of signs and symptoms consistent with sepsis syndrome indicates acute disseminated disease, whereas their absence denotes chronic disseminated disease.

[e] These tests are primarily applicable to aspergillosis and candidiasis and are not useful in diagnosing infections due to *Cryptococcus* species or Zygomycetes (e.g., *Rhizopus, Mucor,* or *Absidia* species). Detection of nucleic acid is not included, because there are as yet no validated or standardized methods.

Note. From "Revised Definitions of Invasive Fungal Disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG)

Consensus Group," by B. De Pauw, T. J. Walsh, J. P. Donnelly, D. A. Stevens, J. E. Edwards, T. Calandra, P. G. Pappas, J. Maertens, O. Lortholary, C. A. Kauffman, D. W. Denning, T. F. Patterson, G. Maschmeyer, J. Bille, W. E. Dismukes, R. Herbrecht, W. W. Hope, C. C. Kibbler, B. J. Kullberg, K. A. Marr, P. Muñoz, F. C. Odds, J. R. Perfect, A. Restrepo, M. Ruhnke, B. H. Segal, J. D. Sobel, T. C. Sorrell, C. Viscoli, J. R. Wingard, T. Zaoutis, and J. E. Bennett (125), 2008, *Clinical Infectious Diseases*, *46*(12), p. 1817 (DOI: 10.1086/588660). Copyright 2008 by Infectious Diseases Society of America. Reprinted with permission.

In contrast, we accepted a culture of a skin tissue sample that was positive for *Aspergillus* to categorize one probable IMI case (i.e., with positive BDG) also as a probable IPA case after discussion of the individual case characteristics. The 2008 EORTC/MSG definitions neither included nor excluded clearly this sample type; however, if the definitions had been interpreted in a strict manner, this case would have been defined as probable IMI and possible IPA.

The remaining cases did not have a host factor or had a host factor but did not meet any clinical criteria; thus, they were classified as unlikely IMI (125).

Radiological Assessment. Besides emergency situations, bronchoscopies were only performed when chest CT scans were available in advance. CT scans were conducted at the Department of Radiology, University Hospital of Graz, and subsequently analyzed by specialists in radiology. These written reports were utilized to assess pulmonary fungal involvement. Investigators verified these reports by analyzing the CT scans themselves when necessary (e.g., when an infectious infiltrate was mentioned, but no further detailed description of its characteristics was made). As delineated in the 2008 EORTC/MSG criteria (125), the clinical criterion was deemed fulfilled if the radiological report included one of the following descriptions:

- dense, well-circumscribed lesions
- air-crescent signs
- cavities.

Microbiological Assessment and Antigen Assays. The Divisions of Hematology, Pulmonology, and Infectious Diseases were involved in the decision-making process regarding performing bronchoscopies. Routine microbiological, histopathological, and

cytological workups of BALF, tissue biopsies, and other sample types (e.g., blood, urine) were performed by the respective laboratories at the Division of Infectious Diseases, Department of Internal Medicine; the Diagnostic and Research Institute for Pathology, Medical University of Graz; the Cytology Institute, University Hospital Graz; and the Institute for Hospital Hygiene and Microbiology, University Hospital Graz.

At our study site, the management of patients with hematological malignancies at risk for IFIs included screening for galactomannan and BDG levels in serum (e.g., twice weekly in general), as well as determination of galactomannan levels in BALF when bronchoscopies were performed. Galactomannan and BGD measurements were carried out at the Institute of Medical and Chemical Laboratory Diagnostics, University Hospital Graz. In cases where (a) serum galactomannan or serum BDG had not been evaluated within clinical routine with samples collected from 24 hours before to 24 hours after bronchoscopy, or (b) BALF galactomannan had not been measured, the respective assays were performed with stored samples at the end of the study (see Figure 4). For measurements of galactomannan levels, the commercially available and widely utilized Platelia *Aspergillus* Ag assay (Bio-Rad Laboratories, Vienna, Austria) was employed. This assay is a double sandwich enzyme immunoassay based on the monoclonal antibody EB-A2 (213). The EB-A2 antibody can detect side chains of galactomannan that consist of at least four β-1,5-galactofuranose residues (45, 293, 294). The cut-off for galactomannan in BALF and serum was defined as 0.5 ODI, as specified by the manufacturer and approved by the US Food and Drug Administration (FDA) (246). Even though the official cut-off for BALF is defined as 0.5 ODI, the ultimately optimal cut-off remains a subject of ongoing discussion. A cut-off of 1.0 ODI is often preferred to other cut-off values between 0.5–1.0 ODI; however, the 2008 EORTC/MSG consensus definitions are related to the recommendations of the manufacturers of the assays (170, 176, 181). Furthermore, the cut-off of 0.5 ODI is also supported by Reinwald et al. (246) who demonstrated that this cut-off level better accounted for the lowered performance of galactomannan in BALF when this test was applied to a population that comprised a high proportion of participants receiving mold-active antifungal agents (234, 246). Given that mold infection prophylaxis is prescribed for high-risk patients at our center, it was to be expected that a high proportion of the study population would be receiving mold-active medications at the time of sample collection.

Serum BDG levels were measured using the Fungitell assay (Cape Cod Diagnostics, Falmouth, MA, United States) which was run on a routine BCS XP coagulation analyzer (295). The cut-off for serum BDG was defined as ≥ 80 pg/mL, in accordance with the manufacturer's recommendations. Values in the range of 60–79 pg/mL are officially considered indeterminate and were interpreted as negative in our study to achieve dichotomous test results. However, BDG was not utilized to classify IMIs when an infection by *Pneumocystis* or *Candida* had been confirmed.

After finishing recruitment, the 2020 EORTC-IDG/MSGERC consensus definitions established cut-offs for galactomannan at 1.0 ODI for individual BALF or serum measurements (127). Lower cut-offs are applicable when both BALF and serum measurements are available: An ODI of 0.8 or higher in BALF can be considered positive if there is an ODI of 0.7 or higher in serum, and vice versa (127). To address this changed situation, a corresponding post-hoc analysis using the 2020 EORTC-IDG/MSGERC consensus definitions was added to the results.

Index Tests

Following a comprehensive review of the available literature and a thorough discussion within our team, we selected the following immunological biomarkers for further evaluation: IL-4, IL-6, IL-8, IL-10, IL-15, IL-17A, IL-22, sIL-2R, IFN-γ, TNF-α, and CCL5 (42, 50, 53, 54, 67, 74-80, 90, 92, 93, 100, 106-114, 117-124, 137, 141, 147, 149, 150, 157). However, it should be noted that this selection is not exhaustive but was limited by the available resources. The references cited are addressed in the introduction. Concentrations of cytokines/chemokines were measured at the Center for Medical Research, Medical University of Graz, with a personalized 11-plex ProcartaPlex immunoassay (eBioscience, Vienna, Austria) in September and October 2016, in May 2017, and in September 2017 (see Figure 4). Until these measurements, samples were stored frozen at –70 °C. The immunoassay consisted of a 96-well plate and was performed in accordance with the manufacturer`s guidelines. The tests were performed with 25 µL of freshly thawed BALF, serum, or lithium-heparin plasma samples including utilization of magnetic beads. Standard curves were calculated for each cytokine by using the manufacturer`s reference solutions. Cytokine/chemokine concentrations were collected with the Bio-Plex 200 system (Bio-Rad, Vienna, Austria) and the Bio-Plex Manager 6.1 software (Bio-Rad, Vienna, Austria). The calculations of concentrations were executed through the implementation of five-parameter logistic curve fitting based on the

standard curves. Concentrations within the standard range were reported as continuous variables in pg/mL. Extrapolated level values below the standard range were defined as not quantifiable and handled as 0.0 pg/mL within the statistical analysis. In rare instances where further dilution of the sample material was not feasible, extrapolated values above the standard curve were defined at the maximum detectable value within the standard curve.

The Department of Hematology and Oncology at Mannheim University Hospital, University of Heidelberg, Mannheim, Germany, performed the nested two-step *Aspergillus* PCR assays as described by Skladny et al. (296) using EDTA whole blood and BALF samples. Samples with a volume of 2 mL were dispatched to Mannheim on the same day of collection via overnight transport. DNA was extracted in accordance with the methodologies outlined by Skladny et al. (296, see Appendix) and Sambrook et al. (297). The amplification of an 138-base pair fragment of the human glucose-6-phosphate dehydrogenase gene (GenBank accession no. X55448) was implemented in the assay as an internal control (296). The PCR was performed utilizing a thermal cycler. The first step primer pair was AFU7S-AFU7AS and the second step primer pair was AFU5S-AFU5AS, matching the 18S rRNA gene sequences of *Aspergillus fumigatus* (GenBank accession no. AB008401) with the following 5'–3' DNA sequences, as published by Skladny et al. (296, Table 1, p. 3867):

- AFU7S: CGG CCC TTA AAT AGC CCG.
- AFU7AS: GA CCG GGT TTG ACC AAC TTT.
- AFU5S: AGG GCC AGC GAG TAC ATC ACC TTG.
- AFU5AS (parentheses indicate degenerate codes): GG G (AG)GT CGT TGC CAA C(CT)C (CT)CC TGA.

Subsequently to the amplification, the products were separated and visualized by gel electrophoresis (296).

After the newly formatted version of the AspLFD (LOT 11215-028-2; OLM Diagnostics, Newcastle-upon-Tyne, United Kingdom) was available, we performed the LFDs with freshly thawed BALF and serum samples in December 2017, in accordance with the manufacturers' instructions (298). Hemorrhagic BALF samples were treated analogous to serum samples, starting with the centrifugation of the sample, followed by addition of 100 µL buffer solution to 50 µL of serum supernatant (i.e., volumetric ratio of 2:1). Next, this mixture was heated, and 100 µL were used to perform the AspLFD. Thus, approximately 33 µL of serum supernatant were ultimately applied to the assay. Conversely, 100 µL of

non-hemorrhagic BALF supernatant was applied directly into the sample well following centrifugation, without undergoing additional pre-processing. The results were read after a period of 10 minutes. The result of the AspLFD were documented semi-quantitatively based on the intensity of the indicator line; however, gradations of the indicator line intensity were irrelevant for our dichotomous assessment (298).

The comprehensive laboratory protocols are provided in the appendix.

Collection and Storage of Samples

The collected sample types encompassed BALF, serum, EDTA whole blood, and lithium-heparin plasma samples. BALF, serum, and whole blood samples were paired by collecting these samples on the same day (i.e., within 24 hours of bronchoscopy). Following the first 14 months of patient recruitment, the array of collected sample types was extended to lithium-heparin plasma samples. These samples were available from 4 days before to 4 days after bronchoscopy if they were ordered for laboratory examinations within clinical routine by the attending physicians.

BALF specimens were collected within clinical routine and portions were sent to different laboratories depending on ordered diagnostic procedures. Commonly, one portion of the BALF was sent to the Clinical Institute of Medical and Chemical Laboratory Diagnostics, University Hospital Graz, for galactomannan level measurements, among other tests. Another portion was commonly sent to the microbiology laboratory of the Division of Infectious Diseases, Department of Medicine, University Hospital Graz, for microbiological diagnostics. In the microbiology laboratory, the remaining BALF was reserved for this study and stored at +4 °C for further processing by the investigators on the same day, after the patient gave informed consent to participate in the study. Investigators mixed the sample using a sample tube shaker and partitioned it to aliquots for storage. One aliquot was stored at +4 °C until it was sent overnight to the Department of Hematology and Oncology, University Hospital of Mannheim, Mannheim, Germany, to perform the *Aspergillus* PCR on the day after bronchoscopy. The remaining aliquots were stored at –70 °C until measurements of cytokines/chemokines and the AspLFD were performed.

Immediately after the patients gave consent to participate in the study, serum and whole blood samples were collected. Serum samples were centrifuged within 30 minutes of collection, subsequently partitioned into aliquots, and stored at –70 °C until further usage. Whole blood samples were not processed further but were stored at +4 °C until they were

shipped overnight to the Department of Hematology and Oncology, University Hospital Mannheim, Mannheim, Germany, to perform the *Aspergillus* PCR, together with the BALF sample aliquot.

Usually, plasma samples were ordered by the attending physicians within clinical routine several times a week and collected by nurses or other authorized members of the ward staff. The samples were transported to the Clinical Institute of Medical and Chemical Laboratory Diagnostics, University Hospital of Graz, via a collective transport service of the hospital. Consequently, the samples remained at room temperature for varying lengths of time until centrifugation. After the laboratory finished the ordered diagnostics, the remaining plasma was routinely stored at +4 °C for a maximum period of 4 days. Following the recruitment of a patient, these residuals were requested by investigators, partitioned into aliquots, and stored at –70 °C for subsequent analysis.

Statistical Methods

The statistical analysis was conducted using IBM SPSS Statistics for Windows, version 29 (IBM Corporation, Armonk, NY, United States). If additional calculations were necessary (e.g., if effect sizes were not implemented in a statistical test procedure in IBM SPSS Statistics for Windows), or for individual graphical illustrations of descriptive statistics, Microsoft Excel for Microsoft 365 MSO (Microsoft Corporation, Redmond, WA, United States) was used. For the inductive statistics, a two-sided significance level α of 5% was specified. By default, asymptotically determined *p*-values were used; only in situations with a small number of cases (e.g., $N < 20$ or chi-square test with expected counts < 5) the exactly calculated *p*-value was computed where possible. The significance level α was not adjusted for multiple testing, except for pairwise post-hoc group comparisons after tests for multiple samples (e.g., pairwise post-hoc group comparisons with Bonferroni correction following a Kruskal-Wallis *H*-test). It is acknowledged that, strictly speaking, such an adjustment of the statistical significance level would have to be carried out, especially since the individual target structures of the tests under evaluation are pathophysiologically related and influence each other (299, 300). To meet requirements for confirmatory statistical testing, the need for adjustment was even greater since parts of the same data have already been evaluated in our previous publications with similar research questions (81, 82, 301). However, the purpose of this study was primarily exploratory and not confirmatory, and the results of inductive statistics should be interpreted as exploratory testing rather than a proof of a hypothesis (299).

The application of the Bonferroni correction to inductive analysis of 13 primary endpoints (i.e., 11 immunological biomarkers, *Aspergillus* PCR, and AspLFD) with double evaluation (i.e., with serum/whole blood and BALF) would necessitate an adjustment of the local significance level of each statistical test to 0.2% in order to maintain the global significance level at 5% (299, 300). If the analyses of the previous publications had additionally been respected, the necessary adjustment of the local significance level of each test according to Bonferroni correction would have implied a local α of 0.08%.

As generally recommended, 95% CIs and effect sizes are listed for further assessment of the relevance and strength of the findings, provided they could be calculated (299, 300, 302, 303). The selection of the effect size measure was based on the review by Tomczak & Tomczak (304); however, it must be noted that the assessment of the strength of the effect sizes in this thesis is only intended as a rough guide. In the field of immunological biomarker research in human infectious diseases, no published threshold values for effect sizes were found that could be used for interpretating their magnitudes.

Missing data was handled as missing; no imputations were made.

For non-normally distributed metric data (e.g., biomarker concentrations), descriptive statistics included the median and its 95% CI as a measure of central tendency (300, 302). Additionally, quartiles, interquartile range (IQR), and minimum and maximum values were reported as measures of dispersion. Boxplots were selected for graphical representation, but these generally require meaningful IQRs to be useful; thus, boxplots were only produced when this aspect was met. The y-axis of boxplots was transformed with an exponent of 0.5 when necessary to ensure the display of outliers and extreme values while simultaneously maintaining the clarity and interpretability of the boxplots. Logarithmic transformation was not chosen because the value 0 occurred regularly in our data sets but is not defined on the logarithmic scale. IBM SPSS Statistics for Windows defined outliers as levels that exceeded 1.5 times the IQR by default, and extreme values as levels that exceeded 3.0 times the IQR.

When two independent samples of non-normally distributed metric data were to be compared (e.g., biomarker concentrations between cases with and without positive virus tests), Welch's *t*-test was utilized only if the number of cases in each group was at least 30 (300). When this assumption was not met, the Mann-Whitney *U*-test (MWU) was performed (e.g., biomarker concentrations between deceased and not-deceased cases) (299, 300, 302). Cohen's *d* was reported as the effect size for Welch's *t*-test, and *r* as the effect size for the

MWU (304). The effect size r was calculated using the equation $r = |z| / n^{1/2}$, with z = standard test statistic of the MWU; and n = total number of observations ((305); (306), as cited by (304)). Cohen's d was considered small with values of .20–.49, moderate with values of .50–.79, and large with values of .80 or higher ((307), as cited by (304)). The interpretation of r is based on that of Pearson's correlation coefficient; thus, r can be considered small when being .10–.30, medium when being .30–.50, and large when being above .50 ((300, 304); (307), as cited by (305)).

For multiple independent samples of non-normally distributed metric data (e.g., comparing cytokine concentrations between unlikely, possible, and probable/proven IMIs), the Kruskal-Wallis H-test was utilized and the effect size η^2 was calculated utilizing the equation $\eta^2 = (H - k + 1) / (n - k)$, with H = Kruskal-Wallis H-test statistic; k = number of groups; and n = total number of observations ((308), as cited by (304, 305)). As Cohen's f and η^2 can be converted into each other, the common grading for Cohen's f could be used alternatively as a rough guide for orientation ((307), as cited by (305, 309, 310)). This would allow an η^2 with .01–.05 to be considered rather small, with .06–.13 as medium, and with .14 and above rather large. However, the calculation of η^2 based on the Kruskal-Wallis H-test result can also produce negative results, which are not actually defined for η^2 (304, 311). They are nevertheless reported as calculated, since a correction to zero would represent a biased value (311). Regarding interpretation, a negative value may indicate, for example, that the true effect is close to zero and the sample size was too small to provide positive values. When the Kruskal-Wallis H-test was significant, pairwise post-hoc tests with Bonferroni correction (as implemented within the Kruskal-Wallis calculation by IBM SPSS Statistics for Windows) were performed subsequently to evaluate which groups significantly differed from each other (299, 300, 302). In the event of significant results, the effect size r was calculated as described above for the MWU.

The Wilcoxon signed-rank test was performed for paired samples (e.g., longitudinal biomarker evaluation) (299, 300, 302). The effect size r was also reported for the Wilcoxon signed-rank test (304).

Scatter plots were generated to evaluate the relationships between two metric variables (e.g., cytokine concentrations and neutrophil count), and the nature of a potential relationship was determined through graphical assessment. If there was a visible monotonic non-linear relationship, Kendall's τ was calculated, which is similarly interpretable like Spearman's

correlation coefficient (viz., grading like Pearson's correlation coefficient) (300, 302). Plasma was obtained in a significantly less standardized manner and was subject to variable storage times at room temperature or under only slight cooling at +4 °C; therefore, the measurements of plasma and serum taken on the day of the bronchoscopy were compared to each other to estimate the reliability of plasma sample analysis. For this purpose, scatterplots and intraclass correlation coefficients (ICC; based on 2-way mixed-effects, single-rating, absolute agreement models) were used (302, 312). The ICC indicates roughly poor reliability when lower than .50, moderate reliability when .50–.75, good reliability when .75–.90, and excellent reliability when higher than .90 (312).

Categorical-nominal data (e.g., results of the PCR and AspLFD) were reported in descriptive statistics as absolute and relative frequencies (300, 302). For inductive statistics, contingency tables were created, and chi-square tests were performed in the case of independent samples with expected counts of at least five in each cell of the contingency table. If the expected counts of a cell were less than five, Fisher's exact test was reported to ensure completeness. However, it is acknowledged that this test is very conservative and that inductive statistics may not be meaningful in such a scenario (300). The ϕ-coefficient was reported as an effect size for the chi-square test in cases of 2 × 2 tables, and Cramer's V was reported as an effect size for the chi-square test in cases of tables that contained more than two rows or columns (304). Effect sizes ϕ and V can be similarly interpreted like r. Odds ratios (OR) were reported as an effect size for Fisher's exact test and, in cases of OR > 1, were interpreted as small when being 1.44 to 2.47, as moderate when being 2.48 to 4.25, and as large when being 4.26 or higher (which is based on thresholds for Cohen's d, see also Chinn (313) and Sánchez-Meca et al. (314)) (300, 302).

The discriminative potential of metric-scaled biomarkers (e.g., cytokines/chemokines) was evaluated by calculating receiver operating characteristic (ROC) curves and the respective areas under the curve (AUC) (299, 300, 302). In general, AUCs in this context can be considered satisfactory with values between .700 and .800 and excellent with values above .800 (299). For the biomarkers that demonstrated discriminative potential, the optimal cut-off was ascertained through the Youden's index. The diagnostic performance values were calculated for the groups unlikely and possible IMI compared to probable and proven IMI, following the approach of Cruciani et al. (274), de Heer et al. (231), and Leeflang et al. (226).

Sensitivities, specificities, positive predictive values (PPV), negative predictive values (NPV), positive LRs, negative LRs, and DORs were obtained using a syntax within IBM SPSS Statistics for Windows which also provided the corresponding 95% CIs (for sensitivity, specificity, PPV, and NPV the generalized linear models module was implemented); the syntax was provided for this purpose by the IBM Corporation (315). Positive LRs of 3.00 and higher were considered to be a useful test for increasing the post-test probability, and positive LRs of 10.00 or higher indicated an particularly powerful test ((302); (316), as cited in (317)). Regarding reduction of post-test probabilities, equivalent negative LRs of 0.33 and lower indicated useful tests, and 0.10 and lower indicated particularly powerful tests. The effect of multiple LRs on post-test probabilities was calculated with an EBMcalc calculator for post-test probabilities from likelihood ratios and multiple test results (developed by Foundation Internet Services, Pittsburgh, PA, United States; provided by UpToDate, Wolters Kluwer, Alphen aan den Rijn, The Netherlands) (318). Qualitative categorization of pre-test and post-test probabilities were adopted from Medow and Lucey (319) and Power et al. (320): very unlikely (i.e., 0%–9%), unlikely (i.e., 10%–33%), uncertain (i.e., 34%–66%), likely (i.e., 67%–90%), and very likely (i.e., 91%–100%); it is important to delineate these from the probabilities defined by the EORTC/MSG and EORTC-IDG/MSGERC.

The degrees of agreement between (a) on the one hand, the index tests, namely, biomarkers with positive findings in the ROC analysis, the *Aspergillus* PCR, and the AspLFD; and (b) on the other hand, certain criteria of the 2008 EORTC/MSG definitions which represented the reference standard, namely, galactomannan, BDG, and certain radiologic findings (i.e., cavities, nodules, consolidations); were assessed using Cohen's κ coefficient, with a κ of .60 or lower interpreted as poor, .61–.80 as good, and over .80 as excellent agreement (302).

Results

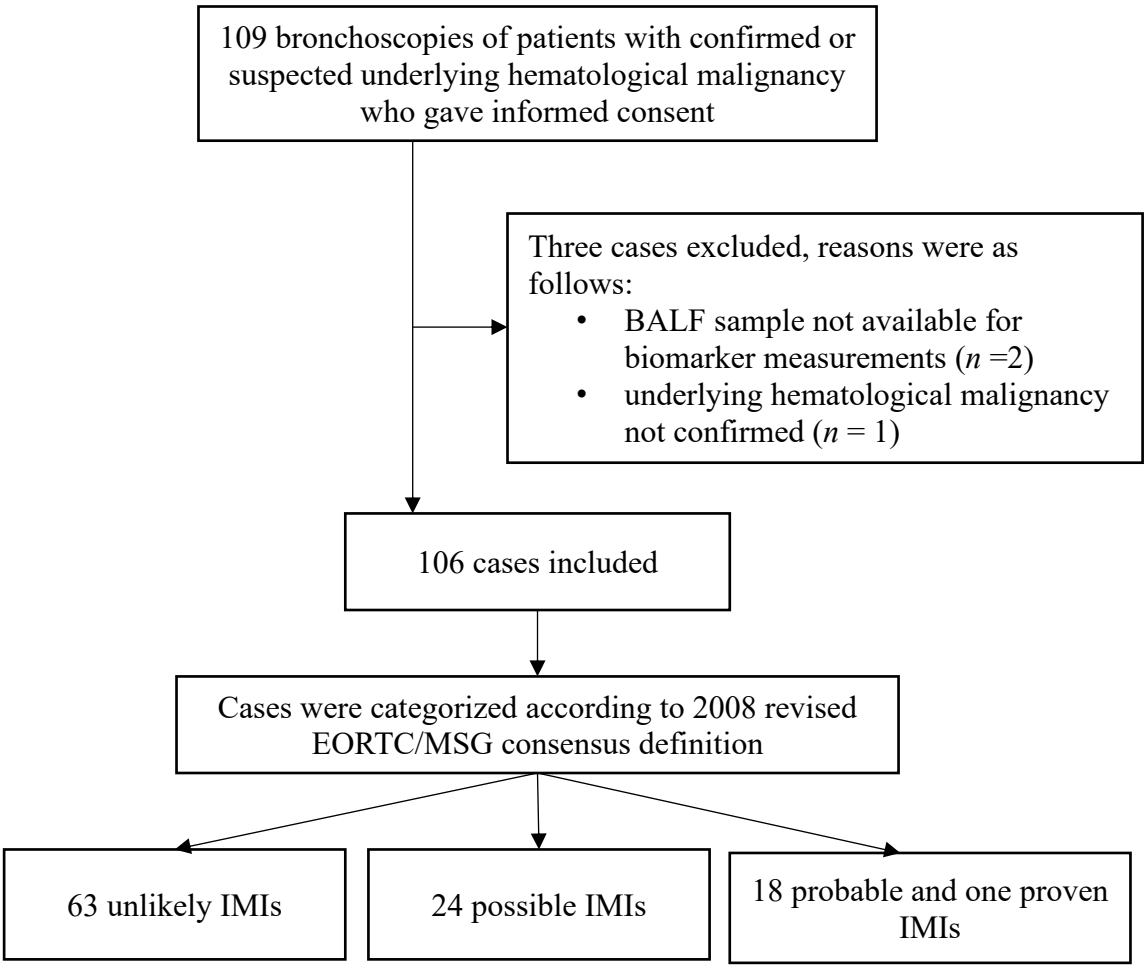
Recruiting Process and Demographic Characteristics

The screening of potential cases for the study commenced when a bronchoscopy for a patient with suspected or known hematological malignancy was scheduled. In approximately 40% of the cases that were screened, bronchoscopy was not performed ultimately. In other approximately 10% of the cases that were screened, the patients declined to participate in the study. The rationales provided by these patients included the following: (a) They refused to undergo any additional bloodwork beyond the clinically necessary ones, (b) they were concerned regarding the potential for compromised data protection, (c) they were concerned about a potential impact of study procedures or results on therapeutic decisions, and (d) they rejected commonly any study participation without a clearly defined rationale.

During the approximately 3-year recruitment period, 109 cases were enrolled. However, three cases were subsequently excluded (see Figure 5): BALF samples of two cases were expended before biomarker measurements had been performed, and in one additional case, the presence of an underlying hematological disease was hypothesized but was not confirmed ultimately. Finally, the statistical analysis included a total of 106 cases. Of the cases included, 59% (63/106) were classified as unlikely, 23% (24/106) as possible, 17% (18/106) as probable, and 1% (1/106) as proven IMI.

Of the probable IMIs, 28% (5/18) exhibited only a positive BDG as mycological criterion, while it was not possible to identify a causative fungal genus (see Table 14 and Figure 6). A total of 61% (11/18) of the probable IMI cases were also assignable as probable IPAs. Microscopic examination of BALF and other respiratory samples did not reveal evidence of molds. In one case, *Aspergillus fumigatus* was cultivated from a skin punch; however, this is a non-sterile material, and subsequent autopsy results did not reveal evidence of a pulmonary mycosis. Regarding respiratory tract samples, molds were identified through culture in only 2% (2/106) of all cases and 11% (2/19) of probable/proven IMI cases, respectively. In both cases, *Scedosporium apiospermum* was identified in tracheal secretions, and in one of these two cases, *S. apiospermum* was also cultured from BALF at a concentration of 10^2 colony-forming units (CFU)/mL.

Figure 5 *Flowchart of Recruitment and Categorization of Cases*



Note. BALF = bronchoalveolar lavage fluid; EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; IMI = invasive mold infection; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

Given that the total number of cultures performed per patient was higher but not precisely documented, it was not possible to calculate the sensitivity of an individual culture conducted with respiratory samples other than BALF; however, the sensitivity would logically have been lower than 11%. Conversely, the total number of BALF samples was known, thereby enabling the calculation of a sensitivity of 5% (1/19) for cultures with BALF samples to detect IMIs. Overall, autopsies were performed in 7% (7/106) of all cases, comprising 28% (7/25) of decedents who passed away prior to discharge.

Table 14 *Characteristics of Probable and Proven Invasive Mold Infection Cases*

Case	Mold-Active antifungals	AspLFD	*Aspergillus* PCR	IL-8 [pg/mL]		IL-6 [pg/mL]
		BALF	BALF	Serum	BALF	BALF
1	Amphotericin B, voriconazole	–	–	5.0	217.6	0.0
2	Amphotericin B	–	–	361.6	2,062.5	73.0
3	Caspofungin	+	–	15.3	263.7	56.3
4	Amphotericin B	–	–	469.1	1,214.3	2,953.2
5	Caspofungin, posaconazole	+	–	808.4	2,087.4	872.4
6	Caspofungin, posaconazole	–	–	61.4	916.1	346.8
7	Caspofungin, posaconazole	–	–	62.0	996.6	194.4
8	Voriconazole	–	–	123.2	1,596.1	1,568.3
9		+++	–	1,328.3	1,015.7	1,222.1
10	Caspofungin	–	–	2,359.2	3,665.0	2,644.6
11	Amphotericin B	++	–	61.3	556.7	29.8
12	Voriconazole	++	+	0.0	5,705.8	144.0
13	Voriconazole	–	–	13.8	965.9	1,163.9
14	Amphotericin B, caspofungin	–	–	4.1	2,079.4	1,215.4
15	Caspofungin	–	–	14.0	2,510.1	656.7
16		–	–	9.2	495.3	33.7
17	Voriconazole	+	+	4.7	4,667.6	180.3
18	Amphotericin B	+	–	14.1	713.4	399.3
19	Caspofungin	+++	+	19.8	1,756.0	196.4

Case	Radiology	Culture being positive for molds	Galactomannan [ODI]			1,3-β-D-Glucan [pg/mL]	
			Serum		BALF	Serum	
			Day of BRSC	Screening [a]		Day of BRSC	Screening [a]
1	Single lesion		0.3	< 0.5	< 0.5	**540.1**	< 80.0
2	Bilateral lesions	Skin punch: *Aspergillus sp.*	0.1	< 0.5	< 0.5	**151.9**	**90.5**
3	Bilateral lesions; cavity		**0.9**	**1.7**	**2.0**	**218.6**	**370.4**
4	Bilateral lesions; cavity		0.3	**0.8**	**0.9**	**150.7**	< 80.0
5	Single lesion		0.4	< 0.5	**1.6**	< 15.4	< 80.0
6	Single lesion		0.1	< 0.5	0.1	**93.8**	< 80.0
7	Single lesion		0.1	< 0.5	0.1	< 15.4	**150.5**
8	Unilateral lesions		0.1	< 0.5	0.5 [c]	**92.2**	< 80.0
9	Bilateral lesions		**1.9**	**1.0**	**4.9**	< 15.4	< 80.0
10	Bilateral lesions		0.1	< 0.5	**1.9**	< 15.4	< 80.0
11	Unilateral lesions		0.1	< 0.5	**0.6**	40.1	**103.4**
12	Bilateral lesions; cavity		0.1	< 0.5	**5.0**	< 15.4	< 80.0
13	Bilateral lesions		0.0	< 0.5	0.1	< 15.4	< 80.0
14	Bilateral lesions	Tracheal secretion: *Scedosporium sp.*	0.1	< 0.5	0.1	**164.0**	**420.7**
15	Bilateral lesions	BALF, tracheal secretion: *Scedosporium sp.*	0.2	< 0.5	0.4	**225.4**	**603.0**
16	Bilateral lesions		0.1	< 0.5	0.1	**305.3**	< 80.0
17	Cavity		0.2	< 0.5	**2.2**	**96.8**	
18	Bilateral lesions; bilateral cavities		0.1	< 0.5	**10.9**	**153.9**	**158.8**
19	Single lesion		0.1	< 0.5	**5.0**	46.5	< 80.0

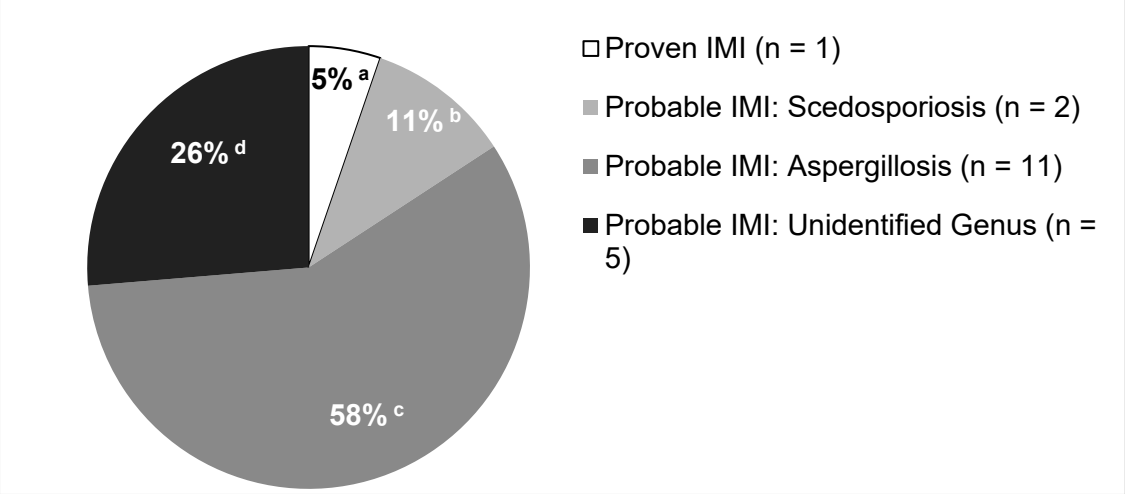
Case	Underlying disease	IMI probability	IPA probability	Host factor
1	NHL	probable	possible	T cell suppression
2	AML	probable	probable [b]	Neutropenia
3	ALL	probable	probable	Neutropenia, T cell suppression
4	ALL	probable	probable	Neutropenia, T cell suppression
5	AML	probable	probable	Neutropenia, T cell suppression
6	AML	probable	possible	T cell suppression
7	AML	probable	possible	Neutropenia, T cell suppression
8	ALL	probable	possible	Neutropenia
9	MM	probable	probable	Neutropenia
10	NHL	probable	probable	T cell suppression
11	NHL	probable	probable	T cell suppression
12	AML	probable	probable	alloHCT, GVHD, T cell suppression, corticosteroids
13	AML	proven [d]	possible	Neutropenia, T cell suppression
14	AML	probable	possible	alloHCT, GVHD, T cell suppression
15	AML	probable	possible	alloHCT, GVHD, T cell suppression
16	AML	probable	possible	alloHCT, GVHD
17	AML	probable	probable	alloHCT, GVHD, T cell suppression, corticosteroids
18	AML	probable	probable	alloHCT
19	MDS	probable	probable	Neutropenia, corticosteroids

Note. The probability of invasive mold infections (IMI) and invasive pulmonary aspergillosis (IPA) was defined according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions (125). Galactomannan (cut-off 0.5 optical density index [ODI] for serum and bronchoalveolar lavage fluid [BALF]) and 1,3-β-D-glucan (cut-off 80.0 pg/mL) were measured on the day of bronchoscopy (BRSC) and in most cases also before and after bronchoscopy as part of a screening setting. The screening tests were indicated and performed as part of clinical care; therefore, the number of tests varied. Values that exceeded the cut offs are formatted in bold. The Aspergillus Lateral Flow Device (AspLFD) yielded semiquantitative results, classified as negative (–), weakly positive (+), moderately positive (++), or strongly positive (+++). The Aspergillus polymerase chain reaction (PCR) yielded dichotomous results, classified as negative (–) or positive (+).

Only the liposomal formulation of amphotericin B was administered. ALL = acute lymphoblastic leukemia; alloHCT = allogenic hematopoietic cell transplantation; AML = acute myeloid leukemia; GVHD = graft-versus-host disease; IL = interleukin; MDS = myelodysplastic syndrome; MM = multiple myeloma; NHL = non-Hodgkin lymphoma.

[a] If screening revealed a positive test, the maximum recorded value is listed. [b] Would have been categorized as possible IPA if a culture from a skin punch had not been accepted as a mycological criterion [c] The non-rounded result was 0.47 ODI, which is why the test was interpreted as negative. [d] Autopsy revealed hyphae in lung tissue.

Figure 6 *Proven and Probable Invasive Pulmonary Mold Infections of the Study Cohort*



Note. The distribution of probable and proven invasive mold infections (IMI) is presented according to mycological evidence. *N* = 19.

[a] Hyphae were identified via microscopy in a pulmonary tissue sample obtained through autopsy; a polymerase chain reaction identified *Cladosporium cladosporioides*. [b] Positive 1,3-β-D-glucan (BDG) along with cultural growth of *Scedosporium* were observed with tracheal secretion samples (n = 2) and with bronchoalveolar lavage fluid (n = 1). [c] Positive galactomannan tests or cultural growth of *Aspergillus* (53% [10/19], if culture from skin punch had not been accepted as a mycological criterion; see also Figure 9). [d] Cases that were assigned to the probable IMI group via BDG, but mold genera remained unidentified (32% [6/19], if culture from skin punch had not been accepted as a mycological criterion).

As determined by autopsies, the underlying causes of death for 5% (3/63) of the unlikely IMIs were identified as (a) multiple organ failure, (b) hemorrhagic pneumonia plus cardiorespiratory insufficiency, and (c) liver failure with following multiple organ failure, respectively. Furthermore, 17% (3/18) of probable IMIs were autopsied, with progressive hematological malignancy and organizing pneumonia (n = 1) as well as organizing viral pneumonia (n = 2) documented as the underlying causes of death. Invasive pulmonary mycosis was documented as the underlying cause of death for the proven IMI case, as the autopsy revealed vital hyphae within the lung tissue accompanied by angio-invasive growth. However, the cultural identification of the causative genus was not initiated by pathologists. Rather, PCR performed on tissue samples identified *Cladosporium cladosporioides/ tenuissimum* (100% homology). Consequently, in accordance with the 2008 EORTC/MSG consensus definitions (125), this case met the criteria for proven IMI but not for proven invasive cladosporiosis. The identification of a genus or species by PCR on tissue samples has been accepted for categorizing cases as proven IMI only since the 2020 EORTC-IDG/ MSGERC consensus definitions (127).

The all-cause 90-day mortality rate was 28% (29/105), whereas 24% (25/105) died during the hospital stay during which they were enrolled in this study; one unlikely IMI case (1/106, 1%) was lost to follow-up (see Table 15 and Figure 7). Among the patients who died during the hospital stay, 32% (8/25) were classified as possible IMIs, while 28% (7/25) were classified as probable/ proven IMIs, including the proven IMI case. Consequently, it could be inferred that IMIs could have been a contributing factor in the fatal outcome of 7%–14% (7/106 to 15/106) of cases in the cohort. The proven IMI case died 50 days after bronchoscopy. One unlikely and three possible IMI cases (4%, 4/105) died after discharge from the hospital stay during which they were enrolled in this study. The relative proportion of deceases was highest in the group of probable/proven IMIs, reaching 37% (7/19). Among the deceased probable/proven IMI cases, 86% (6/7) died within 30 days of bronchoscopy.

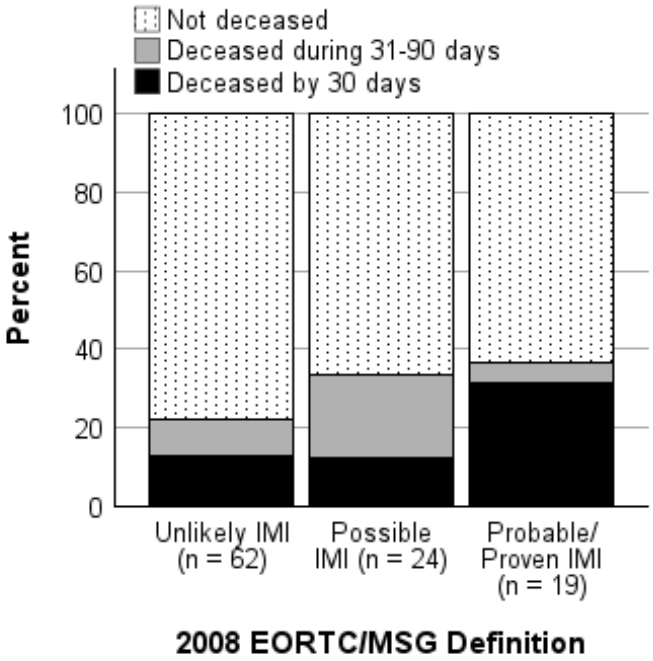
On the day of bronchoscopy, only 11% (2/19) of probable/proven IMI cases (i.e., 18% [2/11] of probable IPA cases) exhibited a positive test result for galactomannan in serum (see Table 16 and Figure 8). Conversely, 58% (11/19) of probable/proven IMIs had a positive test result for BDG in serum on the day of bronchoscopy (see Table 16 and Figure 8).

Table 15 *All-Cause 90-Day Mortality After Bronchoscopy*

Mortality	2008 EORTC/MSG definition						Total	
	Unlikely IMI (n = 62)		Possible IMI (n = 24)		Probable/proven IMI (n = 19)		(N = 105)	
	n	%	n	%	n	%	n	%
90-Day mortality	14	23	8	33	7	37	29	28
30-Day mortality	8	13	3	13	6	32	17	16
Deceased from day 31 to day 90	6	10	5	21	1	5	12	11

Note. Proportions of deceased patients by 90 days after bronchoscopy. One unlikely IMI case was lost to follow-up. EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; IMI = invasive mold infection; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group

Figure 7 *All-Cause 90-Day Mortality After Bronchoscopy*



Note. The ratios of patients who died within 90 days (irrespective of the cause of death) to those who survived. Invasive mold infections (IMI) were categorized in accordance with the 2008 European Organization for Research and Treatment of Cancer/ Invasive Fungal Infections Cooperative Group (EORTC)/ National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) consensus definitions (125). One case from the unlikely IMI group was ultimately lost to follow-up. N = 105.

Table 16 *Galactomannan and BDG Test Results on Day of Bronchoscopy*

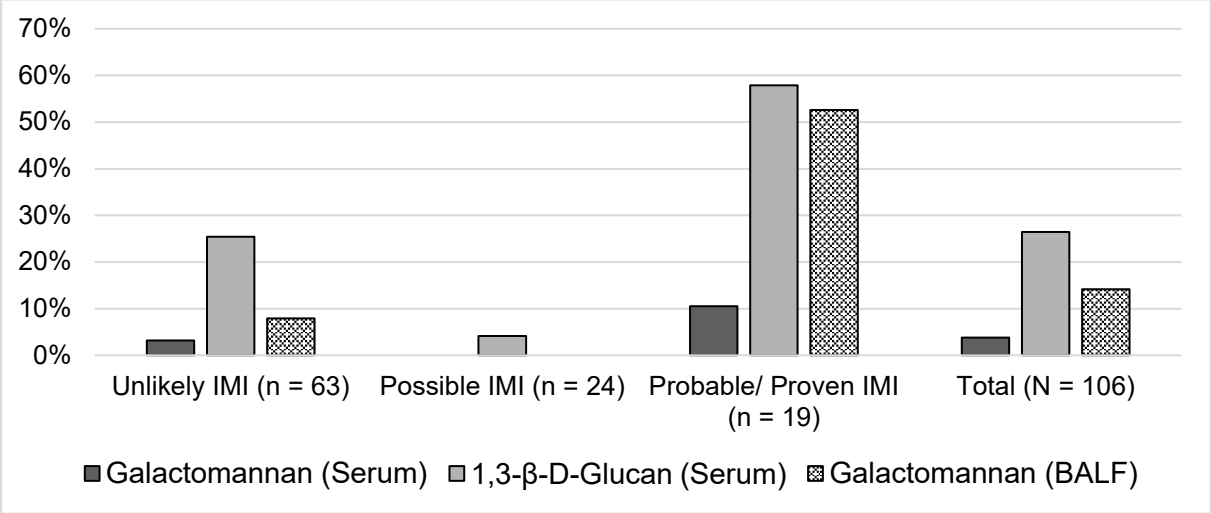
Test	2008 EORTC/MSG Definition			Total
	Unlikely IMI (n = 63)	Possible IMI (n = 24)	Probable/proven IMI (n = 19)	(N = 106)
		Serum		
Galactomannan	2	0 [a]	2	4
	3%	0%	11% [b]	4%
1,3-β-D-Glucan	16	1 [c]	11	28
	25%	4%	58% [b]	26%
		Bronchoalveolar lavage fluid		
Galactomannan	5	0 [d]	10	15
	8%	0%	53% [b]	14%

Note. The frequencies of galactomannan test results in serum and bronchoalveolar lavage fluid (BALF), and of 1,3-β-D-glucan (BDG) test results in serum on the day of bronchoscopy. EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; IMI = invasive mold infection; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

[a] Due to our minor adjustment of the 2008 EORTC/MSG definitions (i.e., the requirement of two positive galactomannan tests with serum for the definition of probable IMI), possible IMIs could have exhibited positive galactomannan tests with serum. [b] Given the utilization of galactomannan and BDG for case classification, these values do not reflect sensitivities. [c] The corresponding BALF sample tested positive for *Pneumocystis* polymerase chain reaction; thus, BDG did not upgrade this case to probable IMI. [d] Because of definition, positive galactomannan tests with BALF were precluded in possible IMIs.

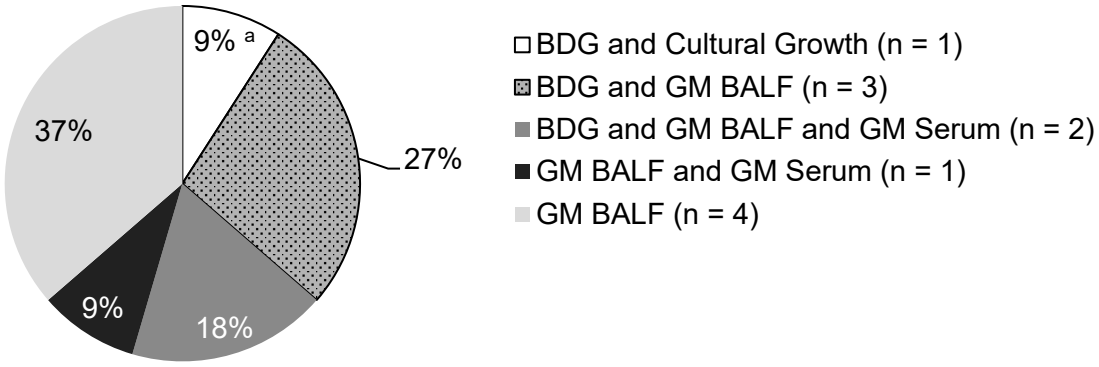
The percentages of positive BDG test results reported in probable/proven IMIs, and of positive galactomannan test results in probable/proven IMIs and in probable IPAs, do not represent sensitivity. These tests were employed for case classification, leading to an incorporation bias with overestimation of the actual sensitivity when assessing test performance.

Figure 8 *Proportions of Positive Galactomannan and BDG Test Results Within Groups*



Note. Proportions of positive galactomannan and 1,3-β-D-glucan (BDG) test results on the day of bronchoscopy. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC)/National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) consensus definitions (125). BALF = bronchoalveolar lavage fluid.

Figure 9 *Mycological Evidence of Cases With Probable Invasive Pulmonary Aspergillosis*



Note. Combinations of positive test results within probable invasive aspergillosis (IA) cases. 1,3-β-D-Glucan (BDG) results were not utilized for defining IA. $N = 11$.
BALF = bronchoalveolar lavage fluid; GM = galactomannan.
[a] One skin tissue sample exhibited the growth of *Aspergillus* in culture, but no evidence of hyphae was reported in the histopathological examination. If the skin punch culture had been excluded for categorizing cases, this case would have been categorized as possible IA and the proportions would have been as follows: 30% (3/10) BDG + GM BALF; 20% (2/10) BDG + GM BALF + GM serum; 10% (1/10) GM BALF + GM serum; 40% (4/10) GM BALF.

However, given that BDG was not utilized for the case classification of the IPAs, the sensitivity and specificity of BDG for detecting probable IPA was calculable, namely, 46% (5/11) sensitivity and 76% (72/95) specificity (i.e., 25% [16/63] of positive BDG tests among the unlikely IPA cases, and 22% [7/32] of positive BDG tests among the possible IPA cases). When including the screening tests before and after the bronchoscopy, 68% (13/19) of probable/proven IMIs (i.e., 55% [6/11] of probable IPA cases) tested positive for BDG in serum at least once, while only 16% (3/19) of probable/proven IMIs (i.e., 27% [3/11] of probable IPAs) tested positive for galactomannan in serum at least once (see Table 14 and Figure 9). Galactomannan tests with BALF yielded positive results in 53% (10/19) of probable/proven IMIs (i.e., in 91% (10/11) of probable IPAs (see Table 16, Figure 8, and Figure 9). As the positive galactomannan tests in BALF upgraded possible IMI cases to probable IMI/IPA cases, there were by definition no possible IMIs with a positive galactomannan test result in BALF.

Candida spp. grew in 22% (23/106) of BALF samples but there was no evidence for invasive candidiasis in any case. Consequently, these findings were interpreted as colonization of the airways or contamination of samples. No blood culture exhibited candidemia, no other yeasts were detected. PCR for *Pneumocystis jirovecii* with BALF was positive in 4% (4/93) of cases (12% [13/106] of cases had not been tested; see Table 17).

For demographic characteristics of the study participants see Table 17. Participants in the possible and probable/proven IMI groups tended to be slightly older compared to the unlikely IMI group; see Figure 10 for a more detailed display of the age distribution. Elevated frequencies within the probable/proven IMI group were also observed for male gender, elevated procalcitonin (PCT), and positive tests for viruses.

Therapies associated with acute myeloid leukemia (AML) are risk factors for the development of IMIs; therefore, the high proportions of AML observed in the possible and probable/proven IMI group were not surprising (see Table 17 and Figure 11). Within the unlikely IMI group, however, AML was also the most frequent underlying disease, narrowly followed by non-Hodgkin lymphoma (NHL).

Like AML, other risk factors for IMIs were more frequently represented within the possible and/or probable/proven IMI group, namely, leukopenia/neutropenia and administration of glucocorticoids or T cell suppressants.

Table 17 *Demographic Characteristics*

Characteristics	2008 EORTC/MSG definition			Total
	Unlikely IMI (n = 63; 59%)	Possible IMI (n = 24; 23%)	Probable/proven IMI (n = 19; 18%)	(N = 106)
Sex				
Male	29	12	12	53
	46%	50%	63%	50%
Female	34	12	7	53
	54%	50%	37%	50%
Age [years] ($M \pm SD$)	56.4	57.3	61.7	57.5
	± 13.0	± 11.0	± 9.2	± 12.0
Underlying hematological malignancy				
Acute myeloid leukemia	22	14	11	47
	35%	58%	58%	44%
Non-Hodgkin lymphoma	19	3	3	25
	30%	13%	16%	24%
Multiple myeloma	7	0	1	8
	11%	0%	5%	8%
Acute lymphoblastic leukemia	5	3	3	11
	8%	13%	16%	10%
Others	10 [a]	4 [b]	1 [c]	15
	16%	17%	5%	14%
Hematopoietic cell transplantation	20	9	9	38
	32%	38%	47%	36%
Allogenic	13	8	6	27
	21%	33%	32%	26%
Autologous	7	1	3	11
	11%	4%	16%	10%
Graft-versus-host disease	7	4	5	16
	11%	17%	26%	15%

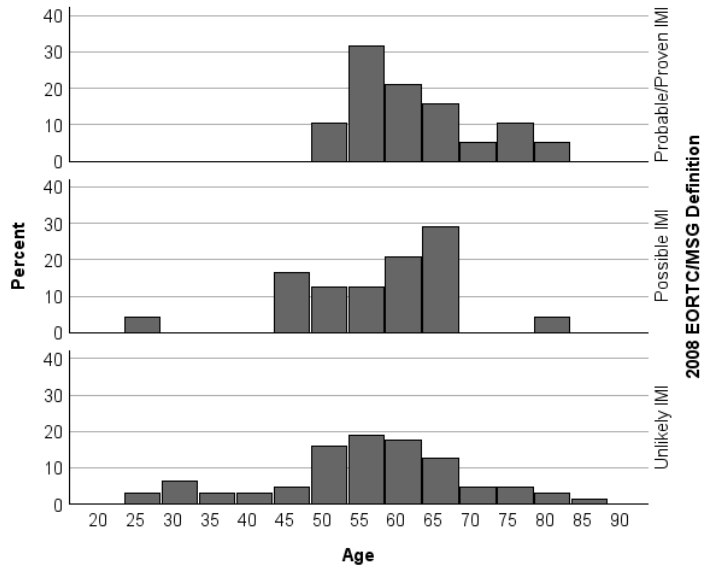
Characteristics	2008 EORTC/MSG definition			Total
	Unlikely IMI	Possible IMI	Probable/proven IMI	(N = 106)
	(n = 63; 59%)	(n = 24; 23%)	(n = 19; 18%)	
Glucocorticoid therapy	18	4	8	30
within ≤ 14 days	29%	17%	42%	28%
Daily ≥ 0.3 mg/kg BW	2	1	3	6
for > 3 weeks [d]	3%	4%	16%	6%
Leukopenia < 4000/µL	29	16	14	59
	46%	67%	74%	56%
Neutropenia < 1500/µL	28	17	13	58
	44%	71%	68%	55%
Neutropenia < 500/µL	14	5	3	22
and ≤ 10 days	22%	21%	16%	21%
Neutropenia < 500/µL	10	10	8	28
and > 10 days	16%	42%	42%	26%
T cell suppressants within	31	19	13	63
≤ 3 months	49%	79%	68%	59%
CRP ≥ 5 mg/L within	54	24	17	95
± 4 days [e]	87%	100%	89%	90%
PCT ≥ 0.5 ng/mL within	14	4	7	25
± 4 days [f]	47%	29%	58%	45%
Positive test for viruses	18	9	11	38
within ± 14 days [g]	31%	39%	61%	38%
Positive test for bacterial infection or parasitosis within ± 14 days [h]	13	8	6	29
	21%	33%	32%	27%
Positive *Pneumocystis jirovecii* PCR with BALF [i]	3	1	0	4
	6%	4%	0%	4%
Culture of BALF with	14	3	6	23
growth of *Candida spp.*	22%	13%	32%	22%

Characteristics	2008 EORTC/MSG definition			Total
	Unlikely IMI (n = 63; 59%)	Possible IMI (n = 24; 23%)	Probable/proven IMI (n = 19; 18%)	(N = 106)
Mold-active antifungal	46	24	17	87
therapy	73%	100%	90%	82%
For ≥ 2 days	45	23	17	85
	71%	96%	90%	80%

Note. Information on time periods refers to the day of bronchoscopy.
BALF = bronchoalveolar lavage fluid; BW = body weight; CLL = chronic lymphocytic leukemia; CRP = C-reactive protein; EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; IMI = invasive mold infection; MDS = myelodysplastic syndrome; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group; PCR = polymerase chain reaction; PCT = procalcitonin; PMF = primary myelofibrosis.
[a] Comprises 6% (4/63) CLL, 3% (2/63) Hodgkin lymphoma and PMF each, and 2% (1/63) aplastic anemia and MDS each. [b] Comprises 8% (2/24) MDS, and 4% (1/24) CLL and PMF each. [c] MDS. [d] Concentration refers to prednisone or an equivalent dose of other glucocorticoid agents. [e] N = 105 (one unlikely IMI case with missing data). [f] N = 56 (33 missing unlikely IMI, 10 missing possible IMI, and seven missing probable/proven IMI cases). [g] N = 99 (five missing unlikely IMI, one missing possible IMI, and one missing probable/proven IMI case). Sample materials included were BALF, blood, pharyngeal swabs, and urine. [h] Bacterial cultures included were conducted with BALF, blood, sputum, and urine. Regarding parasites, one unlikely IMI exhibited a positive *Toxoplasma gondii* PCR with BALF. [i] N = 93 (11 missing unlikely IMI and two missing probable/proven IMI cases).

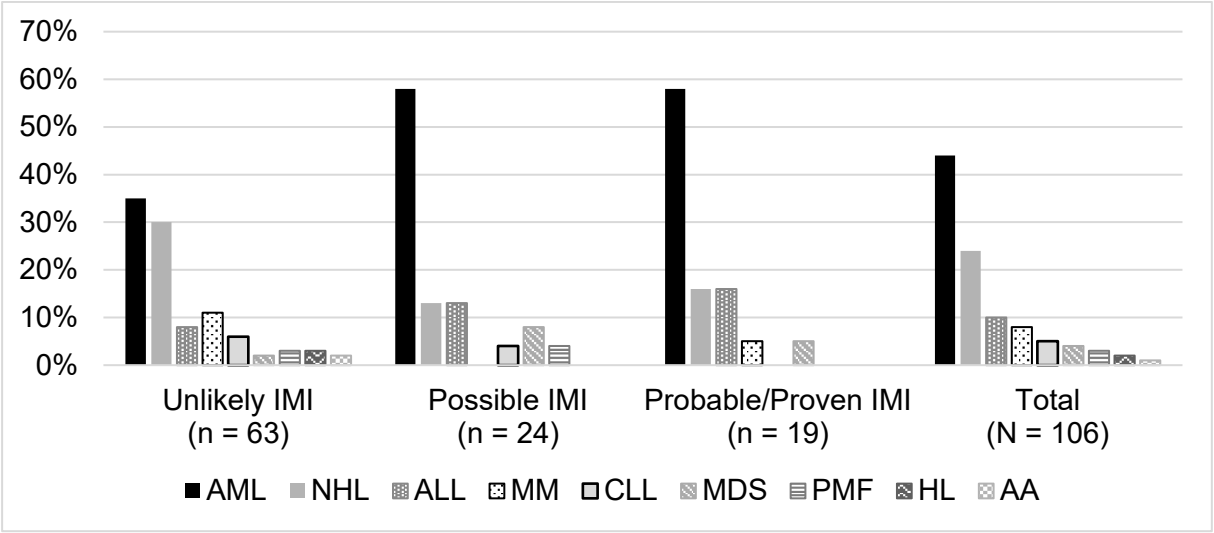
However, neutropenia, treatment with glucocorticoids, or treatment with T cell suppressants were also part of the host factors of the 2008 EORTC/MSG definitions, and at least one host factor was mandatory for categorization of cases into the possible and probable/proven IMI group, leading to higher proportions of host factors within these groups (see also Table 13).

Figure 10 *Distribution of Age*



Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC)/ National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) consensus definition (125). *N* = 106 (63 unlikely IMIs, 24 possible IMIs, and 19 probable/proven IMIs).

Figure 11 *Relative Frequencies of Underlying Hematological Malignancies*



Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definition (125). AA = aplastic anemia; ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; CLL = chronic lymphocytic leukemia; HL = Hodgkin lymphoma; PMF = primary myelofibrosis; MDS = myelodysplastic syndromes; MM = multiple myeloma; NHL = non-Hodgkin lymphoma.

Administered T cell suppressive medications were (see also Figure 12):

- **nucleoside analogues:** cytarabine, fludarabine, clofarabine, decitabine, gemcitabine, azathioprine, or nelarabine in 46% (49/106) of cases.
- **calcineurin inhibitors:** cyclosporine in 9% (10/106) of cases.
- **nucleic acid synthesis inhibitors:** methotrexate or mycophenolate in 9% (10/106) of cases.
- **target of rapamycin (TOR) inhibitors:** sirolimus in 6% (6/106) of cases.
- **specific T cell suppression by monoclonal antibodies:** alemtuzumab in 1% (1/106) of cases.

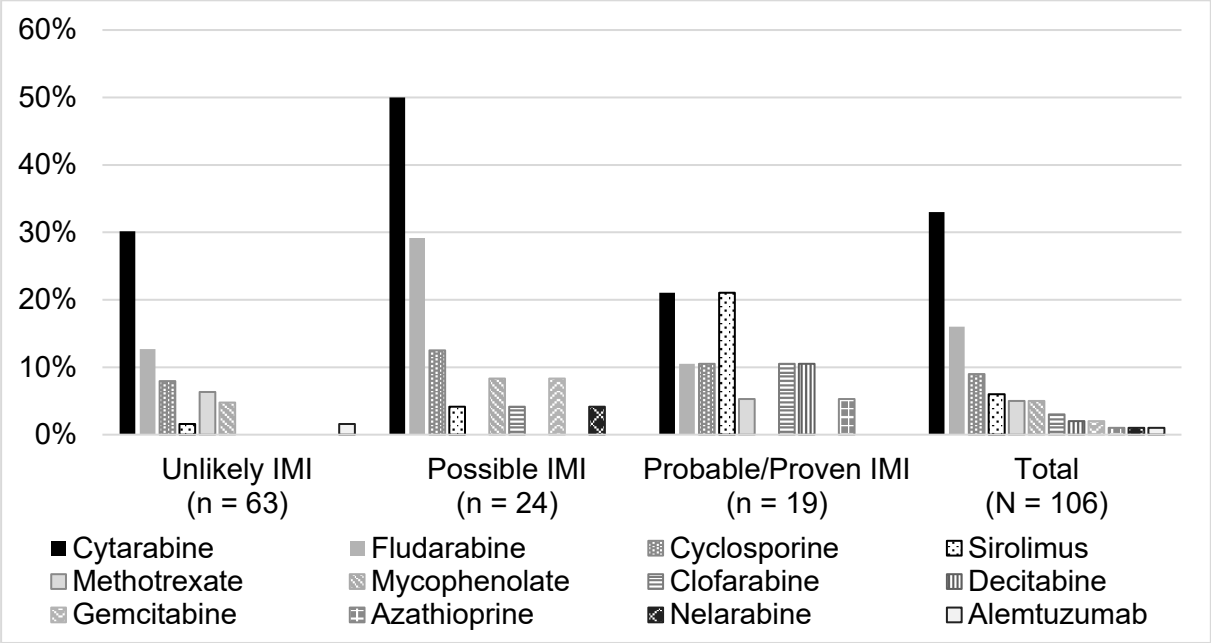
Cytarabine was one of the most frequently prescribed medications in the probable/ proven IMI group, but the other groups contained higher proportions of cytarabine therapy. In contrast, sirolimus, clofarabine, decitabine, and azathioprine were proportionally more frequent within the probable/proven IMI group compared to the other groups.

The glucocorticoid agents administered included dexamethasone (n = 14), prednisolone (n = 11), and methylprednisolone (n = 8).

At the time of bronchoscopy, 82% (87/106) of the patients received mold-active antifungal medications, with 80% (85/106) of patients receiving them for a minimum of 2 days. Antifungal medications were prescribed less frequently among the unlikely IMI cases (see Figure 13). Notably, mold-active antifungal medications were not prescribed to 11% (2/19) of patients in the probable/proven IMI group. Further details to antifungal therapy within our cohort are presented in the chapter Mold-Active Antifungal Therapy.

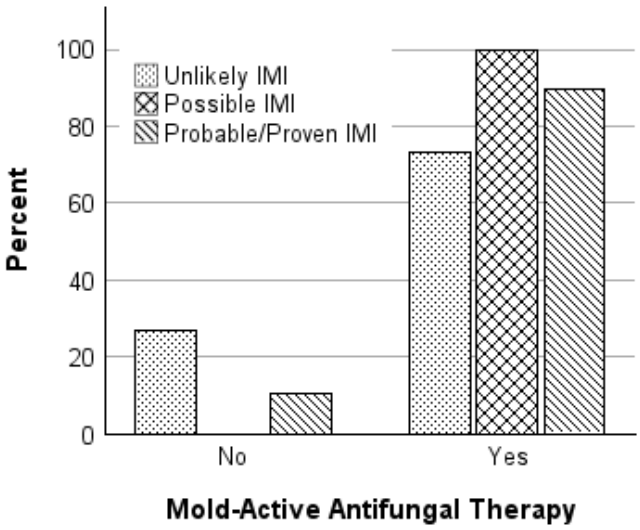
In 15% (16/106) of cases, bacterial species that are commonly regarded as pathogenic in cases of community-acquired or hospital-acquired pneumonias were detected in BALF; the most frequent were *Stenotrophomonas maltophilia* (5/106, 5%), *Pseudomonas aeruginosa* (4/106, 4%), and *Enterobacter spp.* (3/106, 3%; see Figure 14) (321, 322).

Figure 12 *T Cell Suppressing Medication*



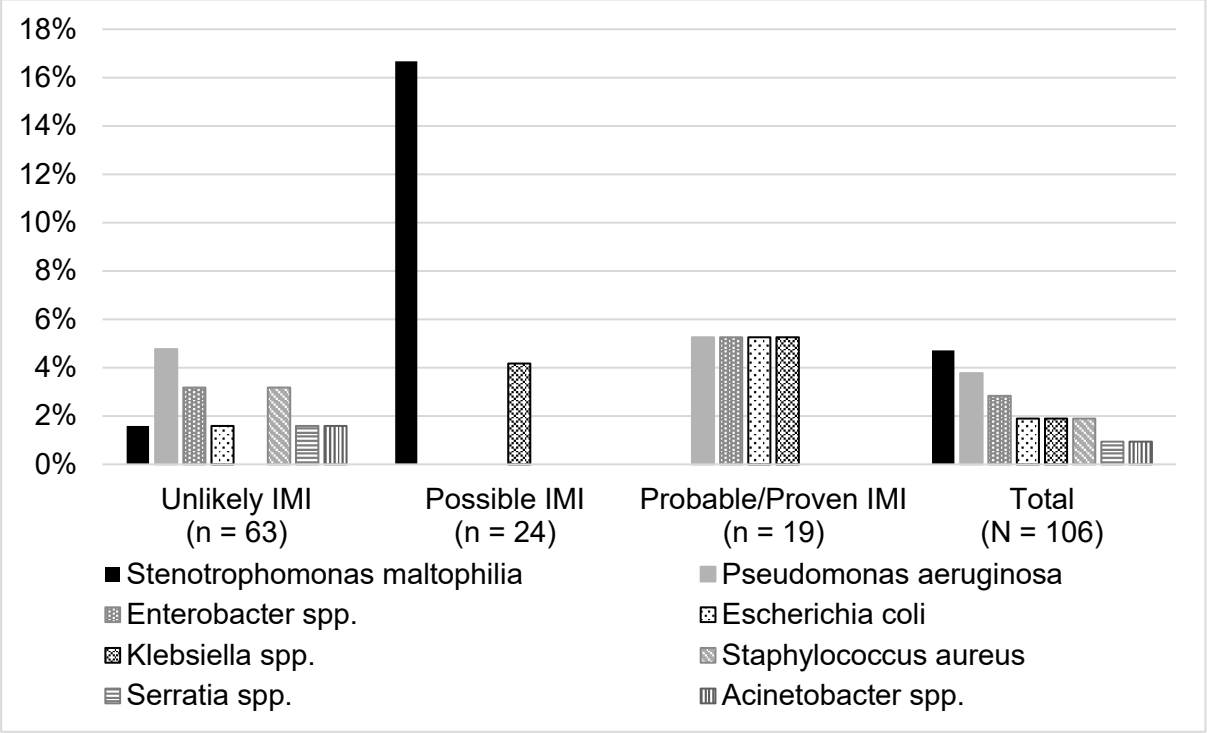
Note. Relative frequencies of T cell suppressing medications administered during a 3-month period preceding bronchoscopy. Invasive mold infections (IMI) were categorized by the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definition (125).

Figure 13 *Antifungal Therapy*



Note. Relative frequencies of cases receiving mold-active antifungal therapies on the day of bronchoscopy. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/ National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions (125). *N* = 106 (63 unlikely IMI, 24 possible IMI, 19 probable/proven IMI).

Figure 14 *Bacterial Growth in Cultures With Bronchoalveolar Lavage Fluid*



Note. Relative frequencies of bacteria that grew in cultures performed with bronchoalveolar lavage fluid. The list includes only bacterial species or genera that are considered potential pathogens for community-acquired pneumonia and hospital-acquired pneumonia. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group definitions (125).

However, the most frequent bacterial pathogens detected in BALF broken down by groups were:

- **probable/proven IMIs:** *P. aeruginosa*, *Enterobacter spp.*, *Escherichia coli*, and *Klebsiella spp.* were isolated with equal frequencies (1/19 [5%] each).
- **possible IMIs:** *S. maltophilia* was particularly frequently identified (4/24, 17%).
- **unlikely IMIs:** *P. aeruginosa* (3/63, 5%), *Enterobacter spp.* (2/63, 3%), and *Staphylococcus aureus* (2/63, 3%) were the most frequently detected bacterial pathogens.

In addition, *Toxoplasma gondii* was detected by PCR in BALF of one unlikely IMI.

During 2 weeks before and after bronchoscopy, 80% (85/106) of cases had blood cultures drawn (i.e., in 50/63 [79%] of unlikely; in 21/24 [88%] of possible; and in 14/19 [74%] of probable/proven IMI cases); bacteremia was verified in eight of these samples:

- **probable/proven IMIs:** One *Pseudomonas sp*., one *Enterococcus sp.*, and one *Klebsiella pneumoniae pneumoniae*; affecting 16% (3/19) of probable/proven IMIs.
- **possible IMIs:** one *Pseudomonas sp*., one *K. pneumoniae pneumoniae*, and one *S. maltophilia*; affecting 13% (3/24) of possible IMIs.
- **unlikely IMIs:** one *Pseudomonas sp.* and one *Enterococcus faecium*; affecting 3% (2/63) of unlikely IMIs.

However, fungemia was not detected in any blood culture.

Urine cultures were obtained from 47% (50/106) of cases during 2 weeks before or after bronchoscopy, and bacterial concentrations of higher than 10^5 CFU/mL were detected in two unlikely (i.e., *E. coli*, *Enterobacter sp.*) and one probable/proven IMI (i.e., *E. coli*).

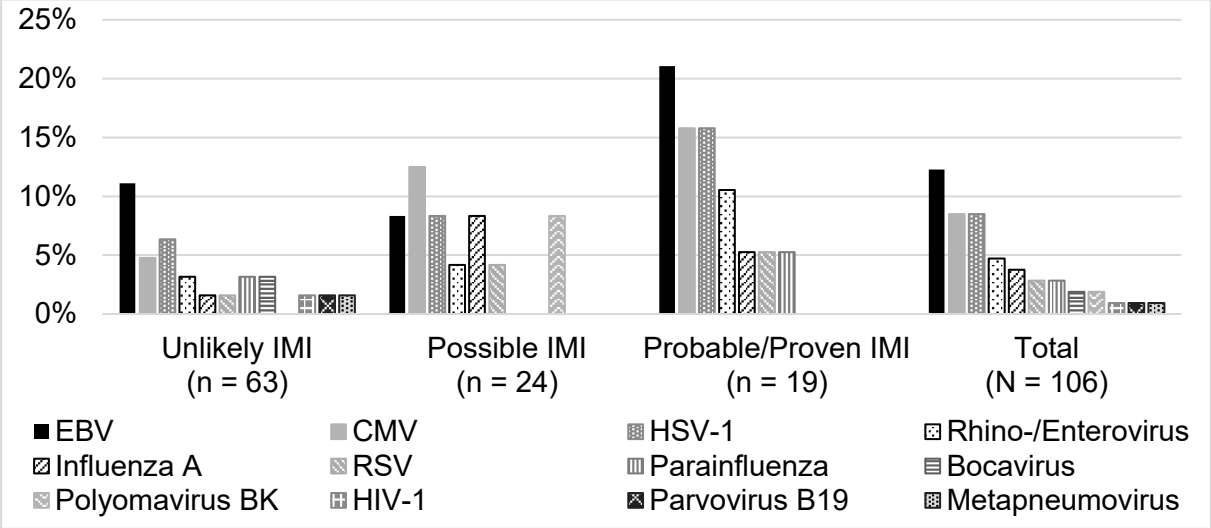
In cultures with sputum or pleural fluid, there were growth of:

- **probable/proven IMIs:** *E. coli* (one case) in sputum.
- **possible IMIs:** *Klebsiella sp.* and *Pseudomonas sp.* (one case each) in sputum.
- **unlikely IMIs:** *Klebsiella spp.* (two cases), *Enterobacter sp.* (one case) and *S. aureus* (one case) in sputum; and *Enterococcus sp.* (one case) and *Staphylococcus epidermidis* plus *Acinetobacter sp.* (one case) in pleural fluid.

During 2-week periods before and after bronchoscopies, viral pathogens were identified in 38% (38/99, seven cases did not undergo viral tests during this period) of cases, and particularly within the probable/proven IMI group. The most prevalent entities were Epstein-Barr virus (EBV; $n = 13$), herpes simplex virus type (HSV) 1 ($n = 9$), and CMV ($n = 9$); these were also the most prevalent entities in probable/proven IMIs (i.e., EBV in 21% [4/19] of probable/proven IMI cases, and HSV-1 and CMV in 16% [3/19] each; see Figure 15). In the unlikely IMI group, EBV was also the most frequently detected replicating virus (i.e., in 11%, 7/63); in the possible IMI group, this was CMV (i.e., in 13%, 3/24).

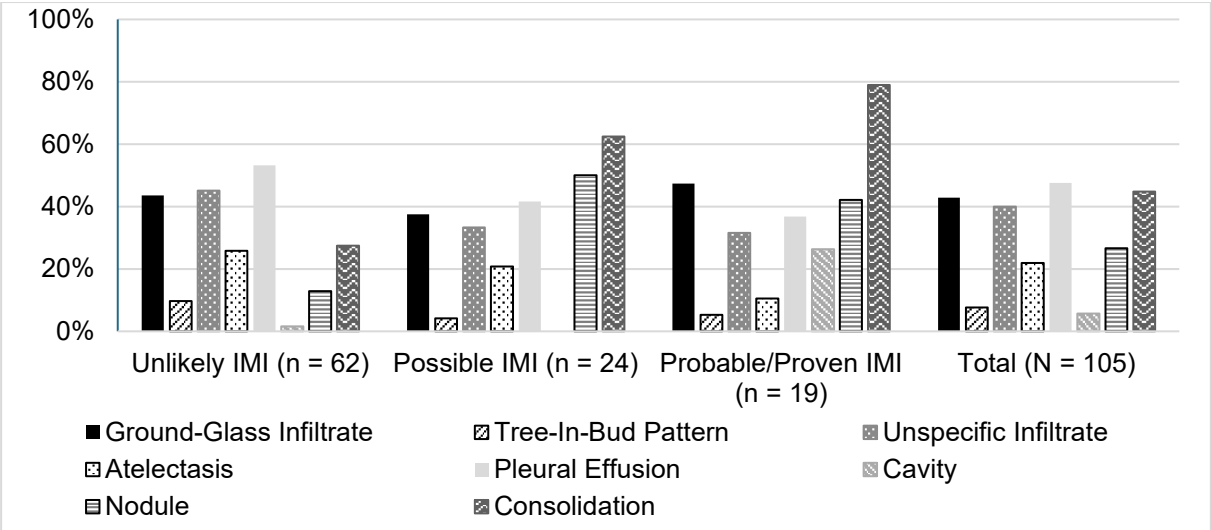
In one case of unlikely IMI (1%, 1/106), the CT of the thorax was not performed. The frequencies of radiologic findings are depicted in Figure 16 and Table 18.

Figure 15 *Relative Frequencies of Viral Pathogens*



Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group definitions (125). CMV = cytomegalovirus; EBV = Ebstein-Barr virus; HIV = human immunodeficiency virus; HSV-1 = herpes simplex virus type 1; RSV = respiratory syncytial virus.

Figure 16 *Relative Frequencies of Radiological Findings in CT Scans of the Thorax*



Note. Invasive mold infections (IMI) where categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group definitions (125). One case within the unlikely IMI group did not undergo a computed tomography (CT) scan of the thorax.

Table 18 *Radiological Findings in Computed Tomography Scans of the Thorax*

Radiological findings	2008 EORTC/MSG definition			Total (N = 105)
	Unlikely IMI (n = 62)	Possible IMI (n = 24)	Probable/proven IMI (n = 19)	
Without intrapulmonary or pleural findings				
	3	0 [a]	0 [a]	3
	5%	0%	0%	3%
Findings not included in the 2008 EORTC/MSG clinical criteria				
Total	57	19	14	90
	92%	79%	74%	86%
Ground-Glass infiltrate	27	9	9	45
	44%	38%	47%	43%
Tree-In-Bud pattern	6	1	1	8
	10%	4%	5%	8%
Other unspecific infiltrates	28	8	6	42
	45%	33%	32%	40%
Atelectasis	16	5	2	23
	26%	21%	11%	22%
Pleural effusion	33	10	7	50
	53%	42%	37%	48%
Findings included in the 2008 EORTC/MSG clinical criteria for lower respiratory tract IMI				
Total	23	24 [a]	19 [a]	66
	37%	100%	100%	63%
Cavity	1	0	5	6
	2%	0%	26%	6%
Dense, well-circumscribed lesions	22	24	18	64
	36%	100%	95%	61%
Halo sign	0	0	0	0
	0%	0%	0%	0%
Air-Crescent sign	0	0	0	0
	0%	0%	0%	0%

Note. One case in the unlikely invasive mold infection (IMI) group did not undergo a computed tomography (CT) scan of the thorax. EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

[a] The presence of radiological findings that were consistent with an IMI was a prerequisite for the definition of a possible or probable/proven IMI.

In 3% (3/105) of cases, no pulmonary or pleural abnormalities were observed that were associated with infectious processes. The radiological findings indicated that 26% (5/19) of the probable/ proven IMI cases had already progressed to an advanced stage, as cavities were present at the time of bronchoscopy. Additionally, the halo sign, an early sign in IPA, was not documented in any case. The most frequently found radiological criterion of the 2008 EORTC/MSG consensus definitions (125) was dense, well-circumscribed lesions. Overall, 27% (28/105) of cases exhibited nodules, and 45% (47/105) exhibited consolidations compatible with an IMI. Most frequent findings in the unlikely IMI group were pleural effusions (53%, 33/62), infiltrates that could not be associated with a specific cause (viz., unspecific infiltrates; 45%, 28/62), and ground-glass infiltrates (44%, 27/62). Possible IMIs most frequently presented consolidations (63%, 15/24) and nodules (50%, 12/24), and probable/proven IMIs also most frequently exhibited consolidations (79%, 15/19).

Immunologic Biomarkers

The levels of the biomarkers were treated as 0.0 pg/mL for statistical analysis when they were below the calibrated measurement range or below the detection limit of the immunoassay. For example, this was the case for IFN-γ in BALF with levels lower than 6.9 pg/mL or 12.4 pg/mL, respectively. The small difference in lower bound levels occurred due to different dates of testing and therefore different calibrations of the assays. Some biomarkers exhibited levels below the calibrated measurement range in a high proportion of cases, as depicted in Table 19. The biomarker concentrations of the sole proven IMI case are listed in Table 20.

Immunologic Biomarkers in Bronchoalveolar Lavage Fluid

Table 21 presents the descriptive analysis of biomarker concentrations within BALF. Most of the values for IFN-γ, IL-10, IL-4, and TNF-α in the BALF were found to be below the calibrated measurement range of the immunoassay. The unlikely IMI group included the highest levels observed for these cytokines. Within the probable/proven IMI group, no IFN-γ levels were detected within the calibrated measurement range.

In a single case of unlikely IMI, an extrapolated IL-6 level of 70,685.1 pg/mL was identified, which exceeded the calibrated measurement range. The measurement could not be repeated, so this value was subsequently changed to 40,000.0 pg/mL (i.e., the upper assay range threshold) for statistical analysis.

Table 19 *Biomarker Levels Below the Calibrated Measurement Range*

Biomarker	Lower bound of calibrated measurement range [pg/mL]			Samples with levels below the calibrated measurement range		
	BALF	Serum	Plasma	BALF (N = 106)	Serum (N = 106)	Plasma (N = 374)
Interferon γ	6.9–12.4	6.3–12.2	7.3–11.9	98%	85%	80%
TNF-α	5.3–6.7	5.2	6.2	89%	97%	92%
IL-10	2.1	2.2	2.2–2.6	93%	66%	50%
IL-15	1.5–11.5	1.2–7.3	2.4–10.0	55%	93%	72%
IL-17A	1.5–1.8	1.4	1.3–2.1	82%	98%	91%
IL-22	18.6	13.5	17.3	33%	87%	61%
IL-4	10.0	10.8	10.8	95%	99%	96%
IL-6	9.0	7.9	7.4	10%	35%	19%
CCL5	0.6	[a]	[b]	4%	1%	0%
IL-8	[b]	2.0	2.0	0%	23%	1%
sIL-2R	69.7–88.2	[b]	[b]	24%	0%	0%

Note. The values given represent the highest extrapolated value below the calibrated measurement range. The slight differences reported for some biomarkers reflect the fact that measurements were taken on different days, thereby necessitating new calibrations.
BALF = bronchoalveolar lavage fluid; CCL5 = cysteine-cysteine motif chemokine ligand 5; IL = interleukin; sIL-2R = soluble interleukin 2 receptor; TNF = tumor necrosis factor.
[a] There was no extrapolated value below the calibrated measurement range, but one value was below the general detection limit of the assay. [b] Each level was within the calibrated measurement range.

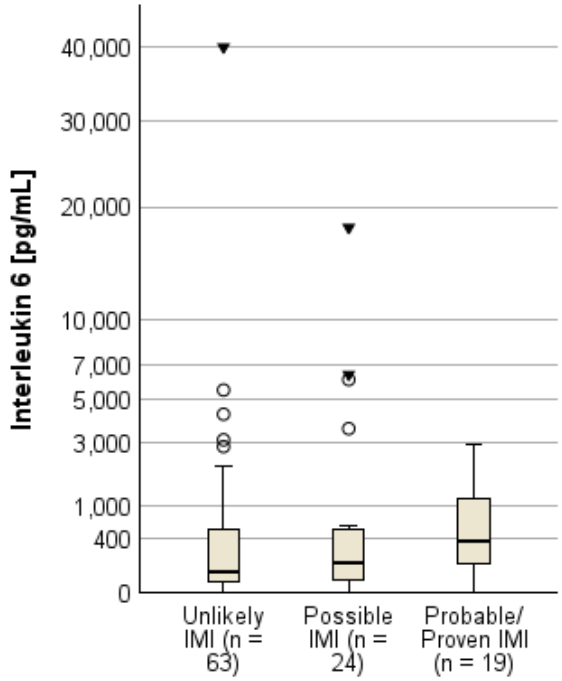
The second highest recorded IL-6 level (17,899.4 pg/mL) was within the calibrated measurement range and belonged to a possible IMI case. The occurrence of additional outliers and extreme values was limited to the unlikely and possible IMI groups, as illustrated in Figure 17. The descriptive analysis revealed a noticeable difference in medians between the unlikely (*Mdn* = 61.10 pg/mL, 95% CI [31.00, 277.27], IQR = 528.2) and probable/proven (*Mdn* = 364.81 pg/mL, 95% CI [143.95; 1,163.89], IQR = 1,142.4) IMI group.

Table 20 *Concentrations of Biomarkers of the Sole Case of Proven IMI*

	Serum [pg/mL]	BALF [pg/mL]
Interferon γ	0.0	0.0
Interleukin 10	0.0	0.0
Interleukin 15	0.0	9.4
Interleukin 17A	0.0	12.4
Soluble interleukin 2 receptor	11,369.6	1,128.6
Interleukin 22	0.0	42.42
Interleukin 4	0.0	0.0
Interleukin 6	0.0	1,163.9
Interleukin 8	13.8	965.9
Cysteine-cysteine motif chemokine ligand 5	25.5	11.2
Tumor necrosis factor α	0.0	0.0

Note. Polymerase chain reaction with lung tissue obtained by autopsy identified *Cladosporium cladosporioides*. BALF = bronchoalveolar lavage fluid; IMI = invasive mold infection.

Figure 17 *Concentrations of Interleukin 6 in Bronchoalveolar Lavage Fluid*



Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). N = 106.

Table 21 *Descriptive Analysis of Biomarker Concentrations in Bronchoalveolar Lavage Fluid*

Statistics	2008 EORTC/MSG definition				Total
	Unlikely IMI	Possible IMI	Probable/proven IMI	*p*	(*N* = 106)
	(*n* = 63)	(*n* = 24)	(*n* = 19)	(η^2)	
			Interferon γ		
Mdn	0.00	0.00	0.00	.596	0.00
95% CI				(–.01)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	0.0	0.0		0.0
IQR	0.0	0.0	0.0		0.0
Min	0.0	0.0	0.0		0.0
Max	251.6	8.8	0.0		251.6
			Interleukin 10		
Mdn	0.00	0.00	0.00	.929	0.00
95% CI				(–.02)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	0.0	0.0		0.0
IQR	0.0	0.0	0.0		0.0
Min	0.0	0.0	0.0		0.0
Max	21.8	3.0	3.2		21.8
			Interleukin 15		
Mdn	0.00	3.21	4.11	.163	0.00
95% CI		[0.00, 7.00]	[4.11, 11.64]	(.02)	
Q1	0.0	0.0	0.0		0.0
Q3	7.0	9.4	7.0		7.0
IQR	7.0	9.4	7.0		7.0
Min	0.0	0.0	0.0		0.0
Max	48.1	38.2	62.1		62.1

Statistics	2008 EORTC/MSG definition				Total
	Unlikely IMI	Possible IMI	Probable/proven IMI	*p*	(*N* = 106)
	(*n* = 63)	(*n* = 24)	(*n* = 19)	(η^2)	
			Interleukin 17A		
Mdn	0.00 $_{a}$	0.00 $_{a, b}$	0.00 $_{b}$	.014	0.00
95% CI				(.06)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	1.7	5.0		0.0
IQR	0.0	1.7	5.0		0.0
Min	0.0	0.0	0.0		0.0
Max	6.3	31.3	12.4		31.3
			Soluble interleukin 2 receptor		
Mdn	216.62	177.24	283.20	.881	239.89
95% CI	[128.78, 358.38]	[113.19, 458.84]	[223.48, 476.00]	(–.02)	[176.53, 315.05]
Q1	76.1	0.0	159.5		94.4
Q3	672.2	754.9	487.4		554.2
IQR	596.1	754.9	327.8		459.8
Min	0.0	0.0	0.0		0.0
Max	40,771.4	19,614.3	1,128.6		40,771.4
			Interleukin 22		
Mdn	42.42	72.01	76.49	.378	42.42
95% CI	[42.42, 101.60]	[0.00, 113.89]	[42.42, 141.42]	(.00)	[42.42, 101.60]
Q1	0.0	0.0	42.4		0.0
Q3	122.7	166.0	174.2		141.4
IQR	122.7	166.0	131.8		141.4
Min	0.0	0.0	0.0		0.0
Max	877.4	797.0	1,217.1		1,217.1

Statistics	2008 EORTC/MSG definition				Total
	Unlikely IMI	Possible IMI	Probable/proven IMI	*p*	(*N* = 106)
	(*n* = 63)	(*n* = 24)	(*n* = 19)	(η^2)	
			Interleukin 4		
Mdn	0.00	0.00	0.00	.442	0.00
95% CI				(.00)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	0.0	0.0		0.0
IQR	0.0	0.0	0.0		0.0
Min	0.0	0.0	0.0		0.0
Max	28.1	11.9	12.1		28.1
			Interleukin 6		
Mdn	61.10	126.77	346.81	.092	103.11
95% CI	[31.00, 277.27]	[32.52, 435.70]	[143.95; 1,163.89]	(.03)	[50.01, 281.07]
Q1	16.0	21.6	73.0		21.5
Q3	544.2	554.9	1,215.4		643.8
IQR	528.2	533.3	1,142.4		622.2
Min	0.0	0.0	0.0		0.0
Max	40,000.0	17,899.4	2,953.2		40,000.0
			Interleukin 8		
Mdn	607.81 $_{a}$	535.75 $_{a, b}$	1,214.27 $_{a}$	.038	718.42
95% CI	[463.03, 839.22]	[339.06; 1,266.51]	[916.11; 2,079.38]	(.04)	[533.13, 965.91]
Q1	257.4	229.4	713.4		339.1
Q3	1,440.0	1,518.8	2,087.4		1,750.9
IQR	1,182.6	1,289.5	1,374.0		1,411.8
Min	58.4	97.5	217.6		58.4
Max	5,672.7	6,754.1	5,705.8		6,754.1

Statistics	2008 EORTC/MSG definition				Total
	Unlikely IMI	Possible IMI	Probable/proven IMI	*p*	(*N* = 106)
	(*n* = 63)	(*n* = 24)	(*n* = 19)	(η^2)	
			Cysteine-cysteine motif chemokine ligand 5		
Mdn	6.70	12.77	7.58	.607	7.82
95% CI	[4.28, 11.10]	[4.02, 29.90]	[4.06, 11.22]	(–.01)	[5.04, 11.01]
Q1	3.1	3.5	3.4		3.3
Q3	18.7	32.9	14.0		23.2
IQR	15.6	29.5	10.6		19.9
Min	0.0	0.0	1.7		0.0
Max	258.7	103.7	37.5		258.7
			Tumor necrosis factor α		
Mdn	0.00	0.00	0.00	.730	0.00
95% CI				(–.01)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	0.0	0.0		0.0
IQR	0.0	0.0	0.0		0.0
Min	0.0	0.0	0.0		0.0
Max	22.8	17.3	19.4		22.8

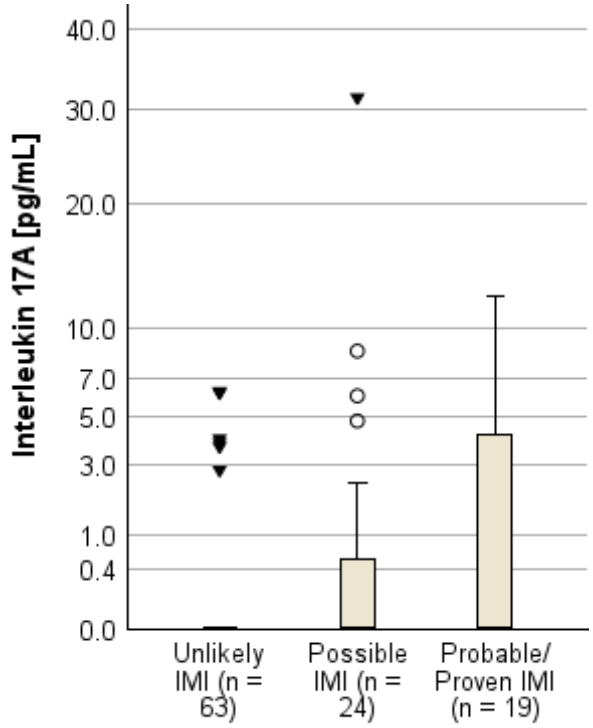
Note. Concentrations are given in pg/mL. The corresponding 95% confidence intervals (CI) listed refer to the medians; however, calculation was not possible in some cases. Differences in the probability distributions of biomarker concentrations between the unlikely, possible, and probable/proven invasive mold infection (IMI) groups were assessed using the Kruskal-Wallis *H*-test with a significance level of α = .05. Correction for multiple testing was only applied for pairwise post-hoc group comparisons (i.e., Bonferroni correction) that was performed when the Kruskal-Wallis *H*-test indicated statistical significance; common subscripts shared within a row indicate that there was no statistically significant difference between the respective groups. In addition to the *p*-value, the effect size η^2 is reported. EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; IQR = interquartile range; Max = maximum; Min = minimum; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group; Q1 = 1st quartile/25th percentile; Q3 =3rd quartile/75th percentile.

However, the 95% CIs of the median of the probable/proven IMI group overlapped half of the 95% CI of the unlikely IMI, and the Kruskal-Wallis *H*-test did not indicate that the probability distributions of IL-6 concentrations between the three groups were different, $H(2, N = 106) = 4.77$, $p = .092$, $\eta^2 = .03$. The effect size was rather small but was comparable to that of IL-8 which gave a significant result with the Kruskal-Wallis *H*-test. The concentrations of the possible IMI group tended to be slightly higher than those of the unlikely IMI group, though not substantial and with similar variability.

The by far highest concentration of IL-17A was observed in the possible IMI group, while the proven IMI case exhibited the second-highest value (see Table 20, Table 21 and Figure 18). The BALF of the possible IMI case yielded a positive result for rhino/enterovirus PCR, and its sputum contained *Pseudomonas sp.* approximately one week prior to bronchoscopy; however, cultures with BALF did not reveal further evidence of a bacterial or fungal infection. Regarding the proven IMI case, microscopy and cultures with BALF also remained negative for pathogens, and tests for viruses yielded negative results. The Kruskal-Wallis *H*-test indicated that the probability distributions for IL-17A concentrations differed slightly to moderately between the three groups, $H(2, N = 106) = 8.54$, $p = .014$, $\eta^2 = .06$. A post-hoc analysis with Bonferroni correction revealed a small difference between probability distributions of the probable/proven IMI and the unlikely IMI group, $z = –2.73$, adjusted $p = .019$, $r = .27$. However, despite that the cases within the probable/proven IMI group exhibited elevated IL-17A levels with greater frequency than within the unlikely IMI group, the median was the same for both groups, *Mdn* = 0.00 pg/mL and IQR = 0.0 for the unlikely IMI group; and *Mdn* = 0.00 pg/mL and IQR = 5.0 for the probable/proven IMI group. This suggests that the Kruskal-Wallis *H*-test and the post-hoc analysis only indicated a different probability distribution, but not a different location of the central tendency of the data.

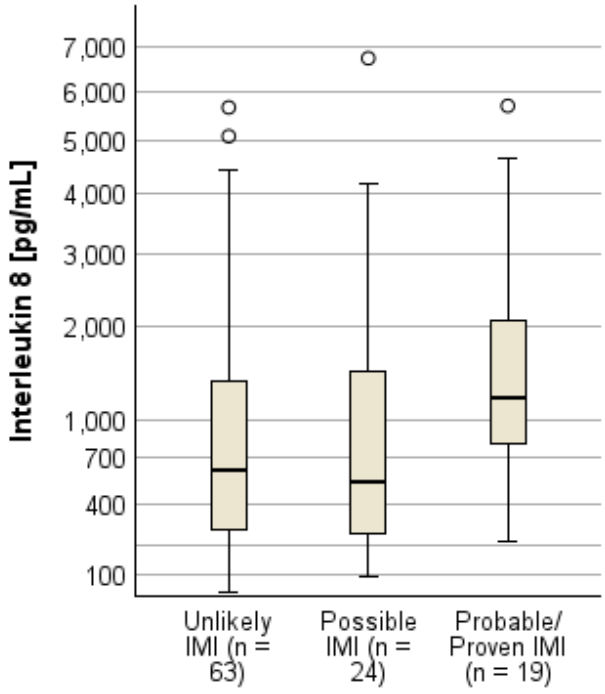
The 95% CI of IL-8 in BALF revealed a difference in the central tendency of the measurements of the unlikely (*Mdn* = 607.81 pg/mL, 95% CI [463.03, 839.22], IQR = 1,182.6) and probable/proven (*Mdn* = 1,214.27 pg/mL, 95% CI [916.11; 2,079.38], IQR = 1,374.0) IMI groups (see Table 21 and Figure 19). Regarding the possible (*Mdn* = 535.75 pg/mL, 95% CI [339.06; 1,266.51], IQR = 1,289.5) and probable/proven IMI group, there was an overlap of the 95% CIs, comprising approximately one third of the 95% CI of the possible IMI group.

Figure 18 *Concentrations of Interleukin 17A in Bronchoalveolar Lavage Fluid*



Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). N = 106.

Figure 19 *Concentrations of Interleukin 8 in Bronchoalveolar Lavage Fluid*



Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots and outliers (○). N = 106.

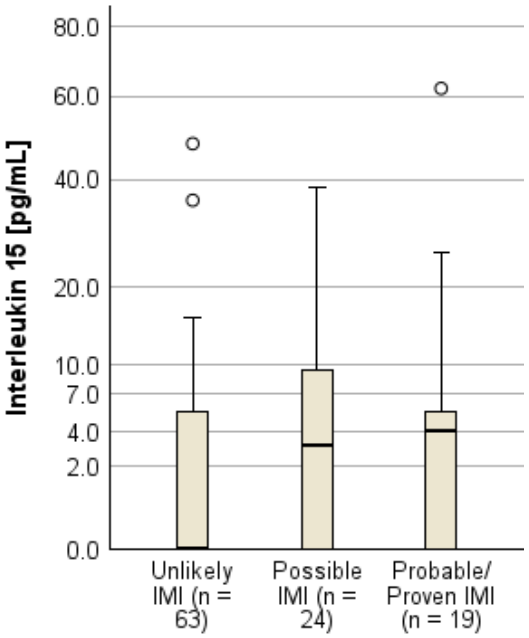
The scattering of measurements was comparable between all three groups. Kruskal-Wallis *H*-test revealed a rather small disparity in probability distribution between the groups, $H(2, N = 106) = 6.54$, $p = .038$, $\eta^2 = .04$. Subsequent post-hoc analysis with Bonferroni correction demonstrated small differences in the probability distributions of IL-8 levels between the probable/proven IMI and unlikely IMI group, $z = –2.468$, adjusted $p = .041$, $r = .24$. While the variability of the data was comparable across both groups, probable/proven IMI cases exhibited higher IL-8 values more likely compared to unlikely IMI cases.

As demonstrated by data in Table 21, Figure 20 (IL-15), Figure 21 (sIL-2R), Figure 22 (IL-22), and Figure 23 (CCL5), these biomarkers did not demonstrate significant different probability distributions or clearly different median concentrations in BALF between the groups. As there were only few cases with biomarker concentrations above 0.0 pg/mL, the boxplots of IFN-γ, IL-10, IL-4, and TNF-α did not yield additional relevant information, consequently, they are not displayed.

ROC analysis for predicting a probable/proven IMI revealed only poor potential for IL-6 and IL-8 in BALF, AUC = .650, 95% CI [.528, .772], $p = .016$; and AUC = .688, 95% CI [.570, .806], $p = .002$, respectively (see Table 22 and Figure 24). IL-17A demonstrated a comparable AUC, though this did not attain statistical significance, AUC = .617, 95% CI [.466, .768], $p = .128$. Additionally, the 95% CIs of the AUCs of IL-17A, IL-22, and IL-15 covered an AUC of .700 or higher which would be considered acceptable for predicting probable/proven IMI. However, the upper bound of the 95% CI of the AUC of IL-8 was .806; therefore, IL-8 was found to be the most promising parameter in BALF.

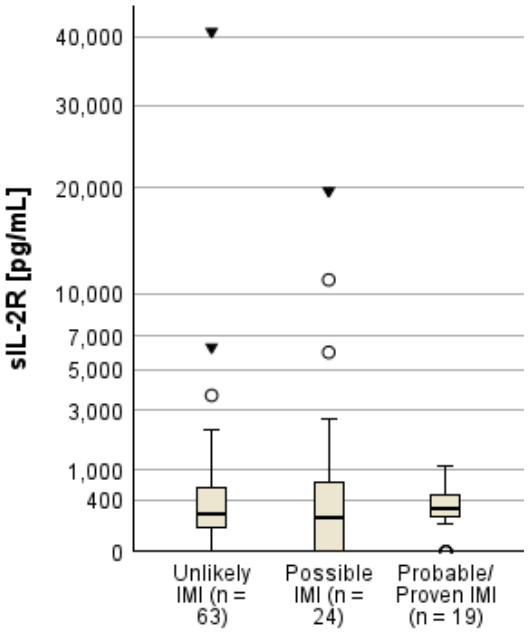
Table 23 presents the characteristics of IL-6 and IL-8 in BALF when utilized as diagnostic tests. The best Youden's index for IL-6 was .34, belonging to a cut-off of ≥ 55.7 pg/mL. There was adequate sensitivity (84%) but low specificity (49%). IL-8 levels ≥ 877.7 pg/mL were associated with a Youden's index of .37, exhibiting 74% sensitivity and 63% specificity. The agreement between IL-6 and IL-8 in BALF when applying these cut-offs was significant but poor, $\kappa = .56$, $p < .001$. Similarly, the comparison of IL-6 and IL-8 in BALF with individual criteria of the 2008 EORTC/MSG consensus definition (i.e., galactomannan, BDG, radiologic criteria for chest CTs) revealed a significant but very poor agreement between IL-6 and galactomannan in BALF, $\kappa = .12$, $p = .048$; and between IL-6 and cavities in chest CT scans $\kappa = .09$, $p = .029$ (see Table 24).

Figure 20 *Concentrations of Interleukin 15 in Bronchoalveolar Lavage Fluid*



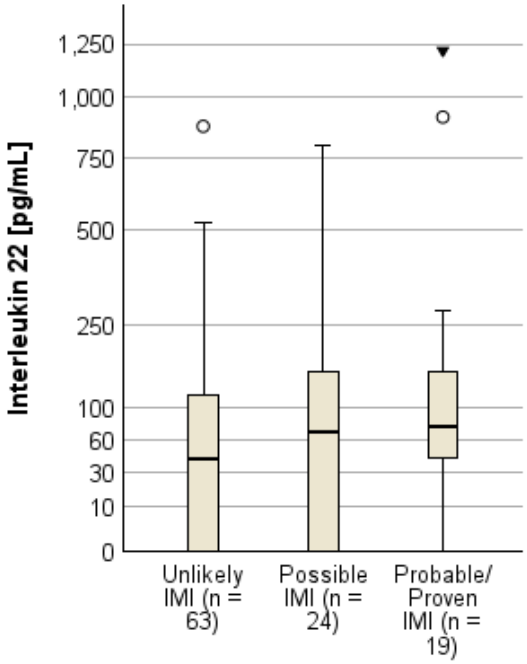
Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots and the outliers (○). N = 106.

Figure 21 *Concentrations of sIL-2R in Bronchoalveolar Lavage Fluid*



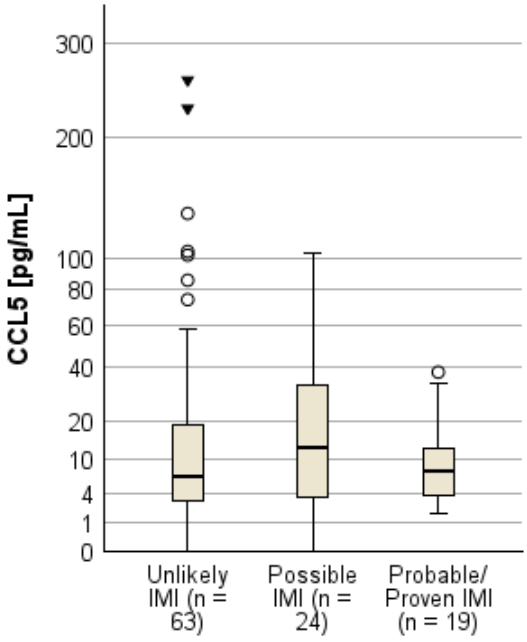
Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). N = 106.
sIL-2R = soluble interleukin 2 receptor.

Figure 22 *Concentrations of Interleukin 22 in Bronchoalveolar Lavage Fluid*



Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). N = 106.

Figure 23 *Concentrations of CCL5 in Bronchoalveolar Lavage Fluid*



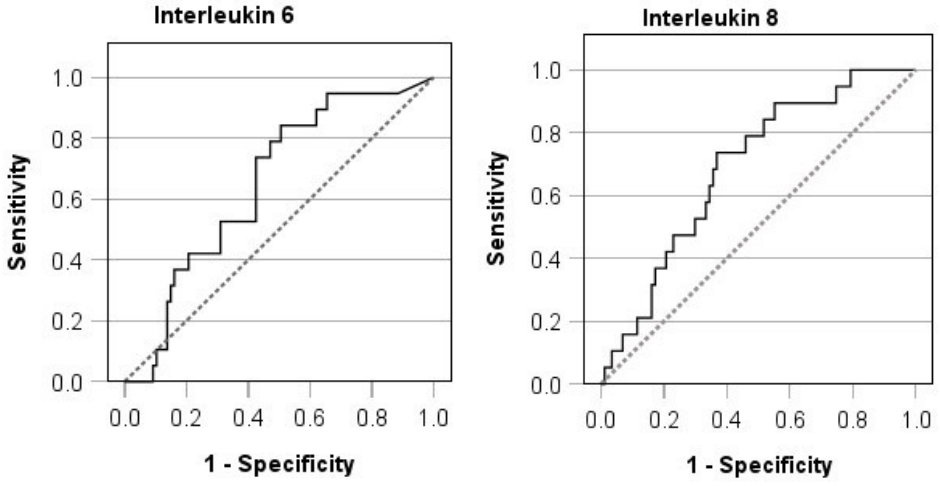
Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots and outliers (○). N = 106. CCL5 = cysteine-cysteine motif chemokine ligand 5.

Table 22 *ROC Analysis for Immunological Biomarkers With BALF Samples*

Biomarker	AUC	*p*	95% CI	
			Lower bound	Upper bound
Interferon γ	.489	.874	.347	.630
Interleukin 10	.492	.917	.350	.635
Interleukin 15	.561	.393	.421	.700
Interleukin 17A	.617	.128	.466	.768
sIL-2R	.537	.566	.412	.661
Interleukin 22	.592	.199	.452	.731
Interleukin 4	.534	.652	.386	.683
Interleukin 6	**.650**	**.016**	**.528**	**.772**
Interleukin 8	**.688**	**.002**	**.570**	**.806**
CCL5	.487	.835	.365	.609
TNF-α	.529	.701	.381	.677

Note. Receiver operating characteristics (ROC) analysis to evaluate the ability to predict probable/proven invasive mold infections (IMI). Results reaching the threshold for significance are displayed in bold. The underlying significance level was defined as α = .05; no correction for multiple testing was performed. *N* = 106 (87 unlikely/possible and 19 probable/proven IMI cases). AUC = area under the receiver operating characteristics curve; BALF = bronchoalveolar lavage fluid; CCL5 = cysteine-cysteine motif chemokine ligand 5; CI = confidence interval; sIL-2R = soluble interleukin 2 receptor; TNF = tumor necrosis factor.

Figure 24 *Receiver Operating Characteristics Curves of Interleukins 6 and 8 in BALF*



Note. Curves refer to the ability to predict probable/proven invasive mold infections (IMI). The diagonal dotted line is the reference line (i.e., area under the curve = .500). *N* = 106 (87 unlikely/possible and 19 probable/proven IMI cases). BALF = bronchoalveolar lavage fluid; IL = interleukin.

Table 23 *Diagnostic Test Performance for IL-6 and IL-8 in Bronchoalveolar Lavage Fluid*

	Diagnostic test characteristics	
		95% confidence interval
Interleukin 6 ≥ 55.7 pg/mL		
Youden's index	.34	
Sensitivity	84%	[64%, 96%]
Specificity	49%	[39%, 60%]
Positive predictive value	27%	[17%, 39%]
Negative predictive value	94%	[84%, 98%]
Diagnostic odds ratio	5.21	[1.42, 19.18]
Positive likelihood ratio	1.67	[1.25, 2.21]
Negative likelihood ratio	0.32	[0.11, 0.92]
Interleukin 8 ≥ 877.7 pg/mL		
Youden's index	.37	
Sensitivity	74%	[52%, 90%]
Specificity	63%	[53%, 73%]
Positive predictive value	30%	[19%, 45%]
Negative predictive value	92%	[83%, 97%]
Diagnostic odds ratio	4.81	[1.59, 14.61]
Positive likelihood ratio	2.00	[1.36, 2.94]
Negative likelihood ratio	0.42	[0.19, 0.90]

Note. Values pertain to the differentiation of probable/proven invasive mold infections (IMI; $n = 19$) from unlikely/possible IMIs ($n = 87$). $N = 106$. IL = interleukin.

Table 24 *Agreement Between Index Tests and Reference Tests*

Test (cut-off)	BALF [a]	Serum [a]		Radiological criteria [b]		
	GM	GM	BDG	Cavity	Nodule	Consolidation
Bronchoalveolar lavage fluid						
IL-6 (≥ 55.7 pg/mL)	.12*	.03	.01	.09*	–.04	.04
IL-8 (≥ 877.7 pg/mL)	.10	.01	.03	.01	.00	.04
AspLFD	.56***	.22**	.07	.43***	.19*	.05
Aspergillus PCR	.24**	.19	–.02	.33**	.04	–.06
Serum						
IL-8 (≥ 55.6 pg/mL)	.18	.03	.01	.00	.00	.03

Note. The values enumerated are κ coefficients. Cases classified as probable/proven invasive mold infection (IMI) were defined as condition positive, while cases classified as unlikely/ possible IMI were defined as condition negative. BALF = bronchoalveolar lavage fluid;

BDG = 1,3-β-D-glucan; GM = galactomannan; IL = interleukin; AspLFD = *Aspergillus* Lateral-Flow Device; PCR = polymerase chain reaction.

[a] $N = 106$ (87 unlikely/possible IMI; 19 probable/proven IMI), except for *Aspergillus* PCR ($N = 101$; 82 unlikely/possible IMI, 19 probable/proven IMI). [b] $N = 105$ (86 unlikely/possible IMI; 19 probable/proven IMI), except for *Aspergillus* PCR ($N = 101$; 82 unlikely/possible IMI, 19 probable/proven IMI).

*$p < .05$. **$p < .01$. ***$p < .001$.

Immunologic Biomarkers in Serum

Table 25 presents the descriptive analysis of cytokine concentrations in serum samples. All IL-4 and TNF-α concentrations of the possible and probable/proven IMI cases and all IL-17A concentrations of the possible IMI cases were below the calibrated measurement range.

The median and quartiles of IL-8 were found to be noticeable elevated for the probable/proven IMI group (*Mdn* = 19.83 pg/mL, 95% CI [13.8, 123.2], IQR = 352.4) in comparison to the unlikely IMI group (*Mdn* = 8.69 pg/mL, 95% CI [5.1, 13.6], IQR = 21.2; see Figure 25). The 95% CIs of these two groups did not exhibit an overlap. Similarly, the 95% CIs of the possible (*Mdn* = 12.79 pg/mL, 95% CI [4.6, 18.7], IQR = 30.5) and probable/proven IMI groups only marginally overlapped. The Kruskal-Wallis *H*-test indicated a slight to moderate difference in probability distributions of IL-8 concentrations in serum between the three IMI probability groups, $H(2, N = 106) = 8.68$, $p = .013$, $\eta^2 = .06$. Subsequent post-hoc analysis with Bonferroni correction revealed that the probable/proven IMI group exhibited slightly to moderately higher levels of IL-8 than the unlikely IMI group, $z = -2.938$, adjusted $p = .010$, $r = .29$.

The other biomarkers revealed no clear differences in concentrations between the groups. The highest individual IL-10 concentrations were exhibited within the possible IMI group (see Figure 26). The highest median of sIL-2R was observed with the possible IMI group. Nevertheless, the highest individual sIL-2R concentrations were measured in the unlikely IMI group, and 95% CIs of medians as well as IQRs of all groups substantially overlapped with each other (see Figure 27). The possible and probable/proven IMI group exhibited higher levels of CCL5 than the unlikely IMI group, but differences were rather negligible (see Figure 28).

Table 25 *Descriptive Analysis of Biomarker Concentrations in Serum*

Statistics	2008 EORTC/MSG definition				Total
	Unlikely IMI (n = 63)	Possible IMI (n = 24)	Probable/proven IMI (n = 19)	p (η^2)	(N = 106)
			Interferon γ		
Mdn	0.00	0.00	0.00	.301	0.00
95% CI				(.00)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	0.0	0.0		0.0
IQR	0.0	0.0	0.0		0.0
Min	0.0	0.0	0.0		0.0
Max	105.8	36.4	14.2		105.8
			Interleukin 10		
Mdn	0.00	0.00	0.00	.145	0.00
95% CI				(.02)	
Q1	0.0	0.0	0.0		0.0
Q3	2.3	4.3	6.8		3.6
IQR	2.3	4.3	6.8		3.6
Min	0.0	0.0	0.0		0.0
Max	142.2	220.2	83.0		220.2
			Interleukin 15		
Mdn	0.00	0.00	0.00	.282	0.00
95% CI				(.01)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	0.0	0.0		0.0
IQR	0.0	0.0	0.0		0.0
Min	0.0	0.0	0.0		0.0
Max	12.9	14.9	14.9		14.9

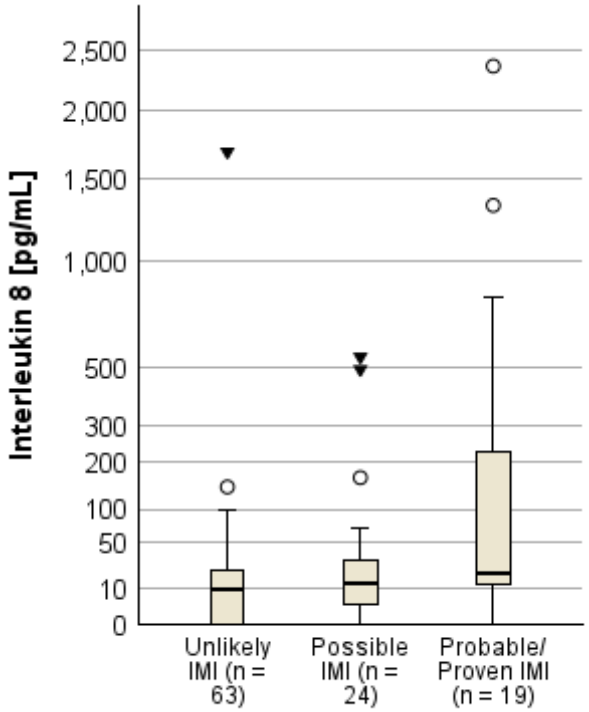
Statistics	2008 EORTC/MSG definition				Total
	Unlikely IMI (n = 63)	Possible IMI (n = 24)	Probable/proven IMI (n = 19)	p (η^2)	(N = 106)
			Interleukin 17A		
Mdn	0.00	0.00	0.00	.431	0.00
95% CI				(.00)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	0.0	0.0		0.0
IQR	0.0	0.0	0.0		0.0
Min	0.0	0.0	0.0		0.0
Max	2.4	0.0	3.7		3.7
			Soluble interleukin 2 receptor		
Mdn	38,428.12	54,767.75	32,554.20	.180	37,738.76
95% CI	[27,107.0; 49,543.4]	[33,946.1; 72,624.9]	[23,676.2; 62,952.0]	(.01)	[32,554.2; 48,197.1]
Q1	14,722.9	31,560.7	13,518.4		18,708.9
Q3	68,745.6	77,973.6	96,575.9		72,085.7
IQR	54,022.7	46,413.0	83,057.5		53,376.8
Min	1,156.4	10,964.6	490.8		490.8
Max	318,362.5	123,522.3	158,267.4		318,362.5
			Interleukin 22		
Mdn	0.00	0.00	0.00	.741	0.00
95% CI				(–.01)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	0.0	0.0		0.0
IQR	0.0	0.0	0.0		0.0
Min	0.0	0.0	0.0		0.0
Max	716.6	140.0	140.0		716.6

Statistics	2008 EORTC/MSG definition				Total
	Unlikely IMI (n = 63)	Possible IMI (n = 24)	Probable/proven IMI (n = 19)	p (η^2)	(N = 106)
			Interleukin 4		
Mdn	0.00	0.00	0.00	.711	0.00
95% CI				(–.01)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	0.0	0.0		0.0
IQR	0.0	0.0	0.0		0.0
Min	0.0	0.0	0.0		0.0
Max	12.8	0.0	0.0		12.8
			Interleukin 6		
Mdn	22.67	19.53	40.24	.261	22.64
95% CI	[8.2, 30.2]	[0.0, 30.4]	[11.6, 200.4]	(.01)	[13.5, 26.7]
Q1	0.0	0.0	11.3		0.0
Q3	77.5	33.1	265.0		119.7
IQR	77.5	33.1	253.7		119.7
Min	0.0	0.0	0.0		0.0
Max	4,384.0	3,044.7	3,049.4		4,384.0
			Interleukin 8		
Mdn	8.69 $_{a}$	12.79 $_{a, b}$	19.83 $_{b}$	.013	10.43
95% CI	[5.1, 13.6]	[4.6, 18.7]	[13.8, 123.2]	(.06)	[8.2, 15.3]
Q1	0.0	2.7	9.2		2.6
Q3	21.2	33.2	361.6		28.1
IQR	21.2	30.5	352.4		25.5
Min	0.0	0.0	0.0		0.0
Max	1,688.8	539.8	2,359.2		2,359.2

Statistics	2008 EORTC/MSG definition				Total
	Unlikely IMI (n = 63)	Possible IMI (n = 24)	Probable/proven IMI (n = 19)	p (η^2)	(N = 106)
	Cysteine-cysteine motif chemokine ligand 5				
Mdn	50.17	57.61	67.01	.114	56.84
95% CI	[42.1, 61.3]	[47.0, 99.4]	[53.7, 109.9]	(.02)	[47.4, 64.7]
Q1	29.7	40.8	40.9		34.0
Q3	78.1	190.4	206.9		93.5
IQR	48.4	149.6	165.9		59.6
Min	0.0	25.2	21.7		0.0
Max	506.8	481.6	608.2		608.2
	Tumor necrosis factor α				
Mdn	0.00	0.00	0.00	.352	0.00
95% CI				(.00)	
Q1	0.0	0.0	0.0		0.0
Q3	0.0	0.0	0.0		0.0
IQR	0.0	0.0	0.0		0.0
Min	0.0	0.0	0.0		0.0
Max	13.3	0.0	0.0		13.3

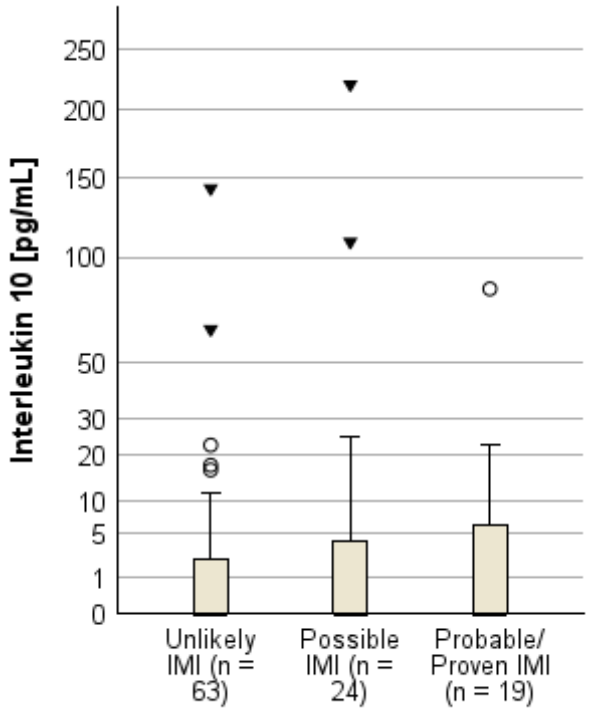
Note. Concentrations are given in pg/mL. The corresponding 95% confidence intervals (CI) listed refer to the medians; however, calculation was not possible in some cases. Differences in the probability distributions of biomarker concentrations between the unlikely, possible, and probable/proven IMI groups were assessed using the Kruskal-Wallis *H*-test with a significance level of α = .05. Correction for multiple testing was only applied for pairwise post-hoc group comparisons (i.e., Bonferroni correction) that was performed when the Kruskal-Wallis *H*-test indicated statistical significance; common subscripts shared within a row indicate that there was no statistically significant difference between the respective groups. In addition to the *p*-value, the effect size η^2 is reported. EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; IQR = interquartile range; Max = maximum; Min = minimum; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group; Q1 = 1st quartile/25th percentile; Q3 =3rd quartile/75th percentile.

Figure 25 *Concentrations of Interleukin 8 in Serum*



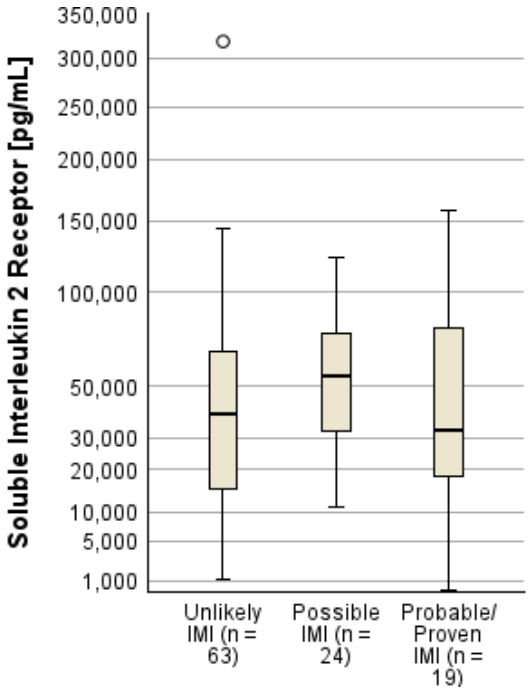
Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). N = 106.

Figure 26 *Concentrations of Interleukin 10 in Serum*



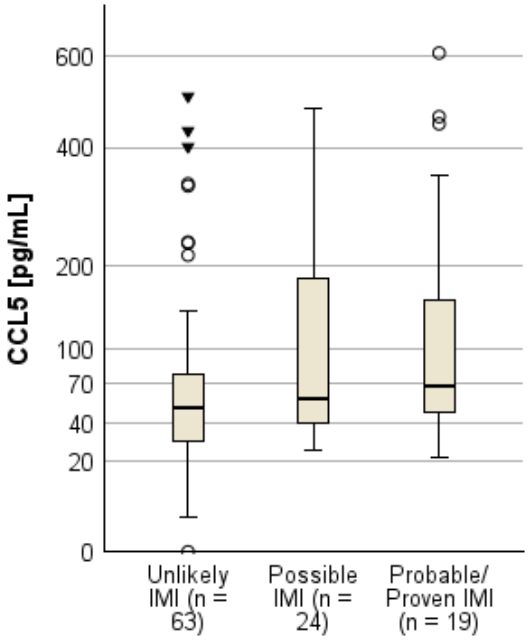
Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). *N* = 106.

Figure 27 *Concentrations of Soluble Interleukin 2 Receptor in Serum*



Note. Invasive mold infections (IMI) were classified by the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been power transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots and the outlier (○). $N = 106$.

Figure 28 *Concentrations of CCL5 in Serum*

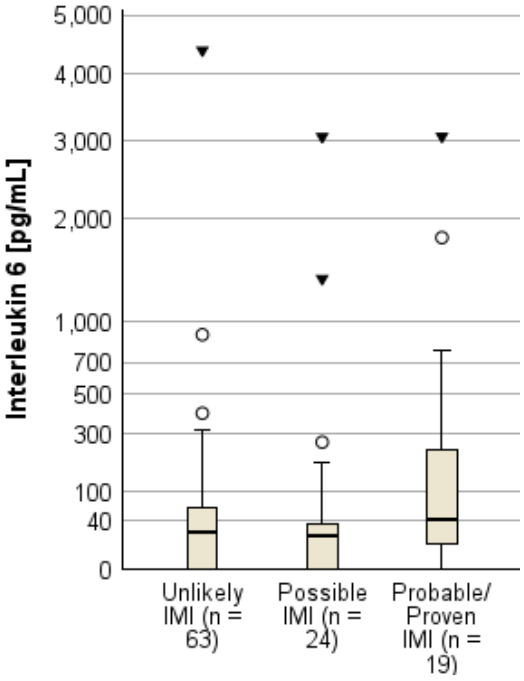


Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). $N = 106$. CCL5 = cysteine-cysteine motif chemokine ligand 5.

The probable/proven IMI group revealed the highest median of IL-6 but also the highest spread of individual concentrations, resulting in a wide 95% CI that overlapped the CIs of the unlikely and possible IMI groups (see also Figure 29). Regarding the remaining evaluated biomarkers in serum, most measured concentrations of IFN-γ, IL-15, IL-17A, IL-22, IL-4, and TNF-α clustered below the calibrated measurement range; thus, their boxplots yielded no further information and were omitted.

Among the selected biomarkers, IL-8 was the only one that exhibited discriminative potential in serum. IL-8 concentrations in serum demonstrated an acceptable potential to predict probable/proven IMI, AUC = .702, 95% CI [.572, .834], p = .002 (see Table 26 and Figure 30). The highest Youden's index (.37) was achieved with a cut-off of ≥ 55.6 pg/mL, resulting in a low sensitivity of 47% and a good specificity of 90% (see Table 27). There was no agreement between IL-8 results using this cut-off and characteristic radiological findings or results from antigen-based tests with serum for IMIs (see Table 24). Other biomarkers demonstrated insufficient discriminatory ability; however, the upper parts of the 95% CIs of the AUCs of IL-10, IL-6, and CCL5 covered a diagnostically relevant range, albeit only narrowly (see Table 26).

Figure 29 *Concentrations of Interleukin 6 in Serum*



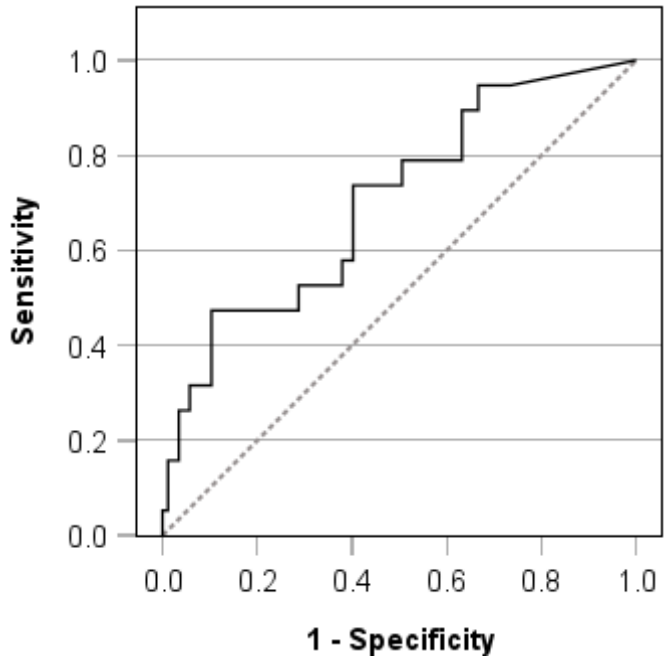
Note. Invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. The y-axis has been transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). N = 106.

Table 26 *ROC Curve Analysis For Immunological Biomarkers With Serum Samples*

Biomarker	AUC	*p*	95% confidence interval	
			Lower bound	Upper bound
Interferon γ	.438	.360	.305	.571
Interleukin 10	.587	.245	.441	.733
Interleukin 15	.549	.519	.399	.699
Interleukin17A	.521	.781	.373	.668
sIL-2R	.461	.626	.304	.618
Interleukin 22	.516	.830	.370	.662
Interleukin 4	.494	.937	.351	.637
Interleukin 6	.617	.112	.473	.762
Interleukin 8	**.703**	**.002**	**.572**	**.834**
CCL5	.599	.195	.449	.748
TNF-α	.483	.810	.342	.624

Note. Receiver operating characteristics (ROC) analysis to evaluate the ability to predict probable/proven invasive mold infections (IMI). The results that reached the threshold for statistical significance are displayed in bold. The underlying significance level was defined as α = .05; no correction for multiple testing was performed. *N* = 106 (87 unlikely/possible and 19 probable/proven IMI cases). AUC = area under the receiver operating characteristics curve; CCL5 = cysteine-cysteine motif chemokine ligand 5; CI = confidence interval; sIL-2R = soluble interleukin 2 receptor; TNF = tumor necrosis factor.

Figure 30 *Receiver Operating Characteristics Curve for Interleukin 8 in Serum*



Note. Curves refer to the ability to predict probable/proven invasive mold infections (IMI). The diagonal dotted line is the reference line (i.e., area under the curve = .500). N = 106 (87 unlikely/possible and 19 probable/ proven IMI cases).

Table 27 *Diagnostic Test Performance for Interleukin 8 in Serum*

Diagnostic test characteristics (cut-off ≥ 55.6 pg/mL)		
		95% confidence interval
Youden‘s index	.37	
Sensitivity	47%	[26%, 69%]
Specificity	90%	[82%, 95%]
Positive predictive value	50%	[28%, 72%]
Negative predictive value	89%	[81%, 94%]
Diagnostic odds ratio	7.80	[2.51, 24.26]
Positive likelihood ratio	4.58	[2.10, 9.98]
Negative likelihood ratio	0.59	[0.38, 0.91]

Note. Values pertain to the differentiation of probable/proven invasive mold infections (IMI; $n = 19$) from unlikely/possible IMIs ($n = 87$). $N = 106$.

Immunologic Biomarkers in Plasma

Plasma sample collection from 4 days before to 4 days after bronchoscopy was incorporated to the study on July 29th, 2015. Consequently, this data was not available for the initial 47/106 (44%) cases. Additionally, missing data occurred for further individual days, because most times blood work was not scheduled daily within clinical routine. Table 28 presents a comprehensive overview of the missing samples for each day of sampling. In the end, 70% (374/531) of possible samples were available from 59/106 (56%) of cases. However, for evaluating changes in biomarker concentrations over time by paired samples, only measurements of samples collected 1 day before and 3 days after bronchoscopy were selected, because this decision ensured that only one probable/proven IMI case dropped out for the statistical analysis; in total, 69% (41/59) of cases for which plasma samples were collected could be included into analysis of paired samples. If only cases had been selected which had had available samples of all days, a mere 17% (10/59; five unlikely, three possible and two probable/proven IMIs) of cases could have been included for analysis.

Due to lack of standardization in the collection of plasma samples in comparison to serum samples, discrepancies of plasma and serum cytokine levels were analyzed for 44 cases of which both sample types were available from the same day (i.e., the day of bronchoscopy). This analysis was undertaken to assess the reliability of the measured plasma concentrations.

Table 28 *Missing Plasma Samples*

Underlying number of cases	Days before BRSC				BRSC	Days after BRSC			
	–4	–3	–2	–1	0	+1	+2	+3	+4
All cases (N = 59)	36	17	15	8	15	13	17	15	21
	61%	29%	25%	14%	25%	22%	29%	25%	36%
Unlikely invasive mold	25	11	9	7	11	10	11	13	15
infections (n = 40)	63%	28%	23%	18%	28%	25%	28%	33%	38%
Possible invasive mold	5	3	3	0	1	1	1	2	3
infections (n = 9)	56%	33%	33%	0%	11%	11%	11%	22%	33%
Probable/proven invasive	6	3	3	1	3	2	5	0	3
mold infections (n = 10)	60%	30%	30%	10%	30%	20%	50%	0%	30%

Note. Missing plasma samples from 4 days before to 4 days after bronchoscopy (BRSC). No plasma samples were drawn exclusively for the study; instead, leftover material from plasma samples taken as part of routinely scheduled clinical procedures was utilized. However, only 10 cases had blood drawn daily (i.e., five unlikely, three possible and two probable/proven invasive mold infections).

Regarding plasma samples, there was greater variability in time (a) until centrifugation, (b) until cooling of the samples at +4 °C, and (c) until archiving of the samples at –70 °C. Table 29 presents the median differences in measurements between both sample types for each biomarker. Scatter plots and ICCs were utilized to check whether there was reproducibility of the measured serum concentrations in the plasma samples despite the deviations in processing and storage. Consulting the scatterplots, IFN-γ (see Figure 31), IL-10 (see Figure 32), and sIL-2R (see Figure 33) levels were reproduced with an acceptable variation in most cases. Regarding an ICC analysis, only IFN-γ and IL-10 had fair to excellent reliability to produce similar values with plasma and serum samples (see Table 30). However, the concentrations of sIL-2R were higher on average in plasma, while the ICC indicated moderate reliability of the measurements, albeit with a certain degree of uncertainty in the form of a wide 95% CI. Comparison of the IL-8 measurements was fairly convincing according to the ICC analysis, but the according scatter plot did not indicate adequate reliability of the measurements (see Figure 34).

Table 29 *Differences in Biomarker Concentrations Between Plasma and Serum Levels*

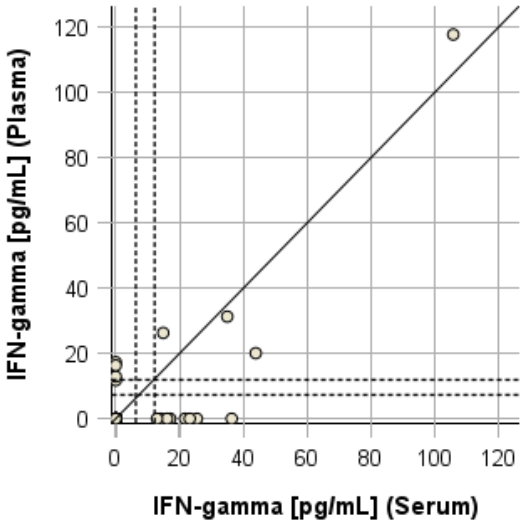
Biomarker	Median difference [pg/mL]	IQR
Interferon γ	0.00	0.0
Interleukin 10	0.00	3.2
Interleukin 15	0.00	0.0
Interleukin 17A	0.00	0.0
Soluble interleukin 2 receptor	–12,433.39	23,913.1
Interleukin 22	0.00	74.5
Interleukin 4	0.00	0.0
Interleukin 6	–0.30	72.3
Interleukin 8	–33.20	78.5
CCL5	–94.89	140.5
Tumor necrosis factor α	0.00	0.0

Note. Comparison of plasma and serum concentrations collected on the day of bronchoscopy. The plasma samples were obtained using less standardized procedures and storage conditions during clinical routine. The serum samples were obtained in a standardized manner by the investigators. The individual differences were calculated by subtracting the plasma concentrations from the serum concentrations, so a negative median indicates a tendency toward higher plasma concentrations, and vice versa. $N = 44$. CCL5 = cysteine-cysteine motif chemokine ligand 5; IQR = interquartile range.

Finally, concentrations of IL-15 (see Figure 35), IL-17A (see Figure 36), IL-22 (see Figure 37), IL-4 (see Figure 38), IL-6 (see Figure 39), CCL5 (see Figure 40), and TNF-α (see Figure 41) did not seem to be reliable in plasma samples after the less standardized sample processing. Therefore, only IFN-γ, IL-10, and sIL-2R are considered in the following analysis and interpretation of the longitudinal profile of biomarker concentrations, whereby results for sIL-2R were considered only limitedly reliable.

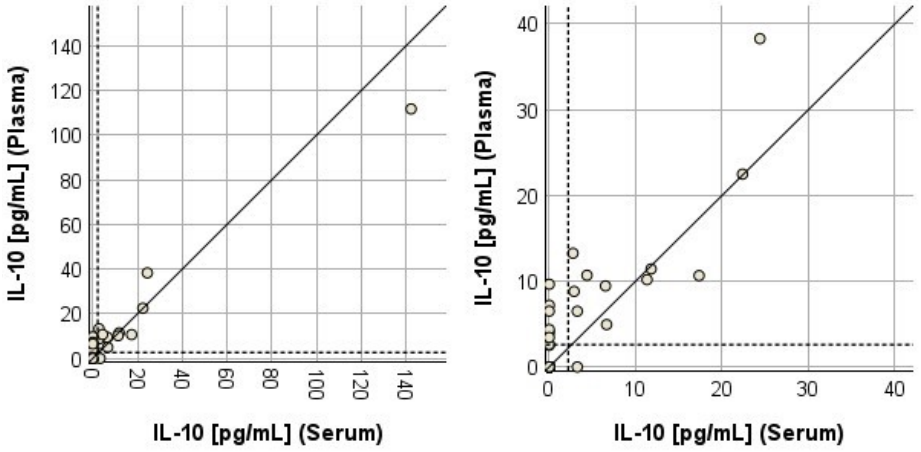
On average, levels of IFN-γ and IL-10 stayed stable. While sIL-2R concentrations tended to increase in the group of possible IMIs, they decreased in the groups of unlikely and probable/proven IMIs. However, these changes were rather marginal.

Figure 31 *Comparison of Concentrations of Interferon γ in Plasma and Serum Samples*



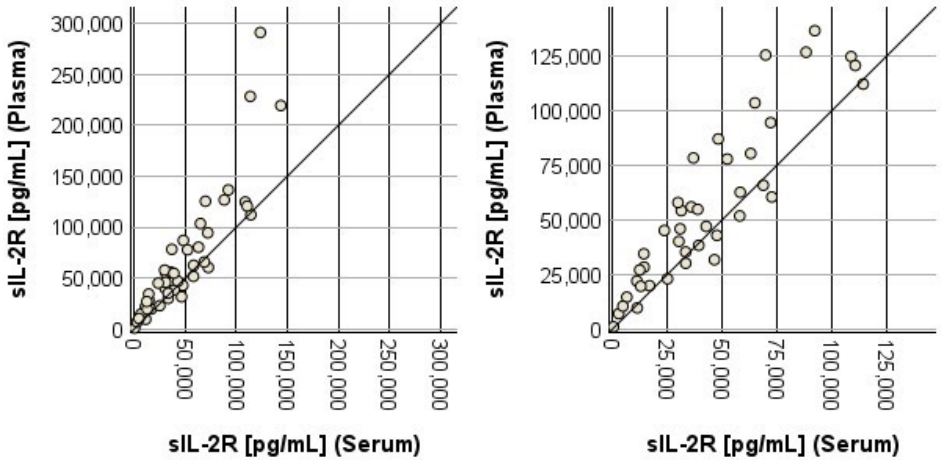
Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). The vertical dashed lines represent the lower bounds of the calibrated measurement range for serum levels (i.e., x = 6.3 pg/mL and x = 12.2 pg/mL). The horizontal dashed lines represent the lower bounds of the calibrated measurement range for plasma levels (i.e., y = 7.3 pg/mL and y = 11.9 pg/mL). The disparity in calibrated measurement range bounds can be explained by different dates of sample analysis. Levels that fell below these thresholds were assigned a value of 0.0 pg/mL. *N* = 44. IFN = interferon.

Figure 32 *Comparison of Concentrations of IL-10 in Plasma and Serum Samples*



Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). The vertical dashed line represents the lower bound of the calibrated measurement range for serum levels (i.e., x = 2.2 pg/mL). The horizontal dashed line represents the lower bound of the calibrated measurement range for plasma levels (i.e., y = 2.6 pg/mL). Levels that fell below these thresholds were assigned a value of 0.0 pg/mL. The scatter plot on the right is identical to the lower concentration range of the scatter plot on the left; the scale has been changed to present the lower concentration range in a meaningful way, at the expense of charting outliers. *N* = 44. IL = interleukin.

Figure 33 *Comparison of Concentrations of sIL-2R in Plasma and Serum Samples*



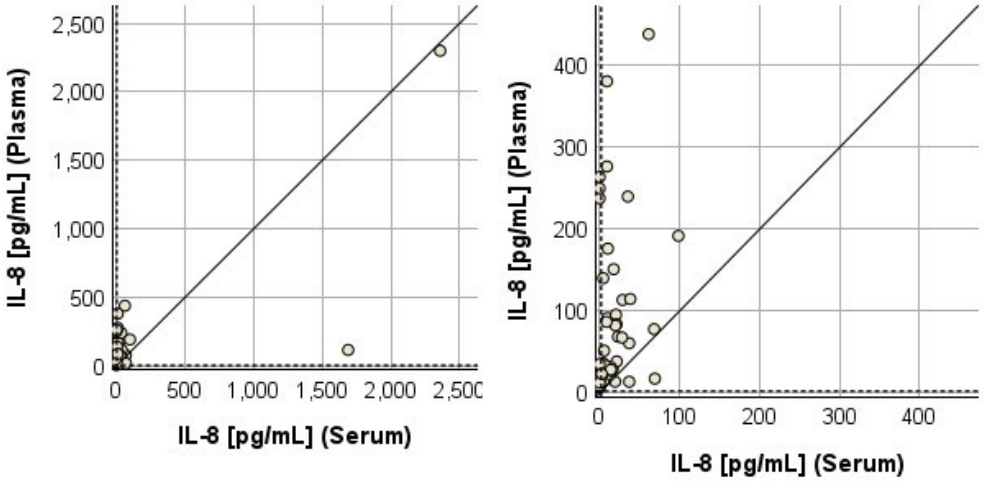
Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). The scatter plot on the right is identical to the lower concentration range of the scatter plot on the left; the scale has been changed to present the lower concentration range in a meaningful way, at the expense of charting outliers. $N = 44$. sIL-2R = soluble interleukin 2 receptor.

Table 30 *ICCs for Biomarker Concentrations in Serum and Plasma*

Biomarker	Intraclass correlation coefficient	95% confidence interval
Interferon γ	.83	[.70, .90]
Interleukin 10	.95	[.92, .97]
Interleukin 15	–.01	[–.28, .27]
Interleukin 17A	.22	[–.06, .47]
Soluble interleukin 2 receptor	.73	[.44, .87]
Interleukin 22	.03	[–.25, .32]
Interleukin 4	–.03	[–.33, .27]
Interleukin 6	.26	[–.03, .52]
Interleukin 8	.76	[.61, .86]
CCL5	.37	[.03, .62]
Tumor necrosis factor α	.14	[–.17, .42]

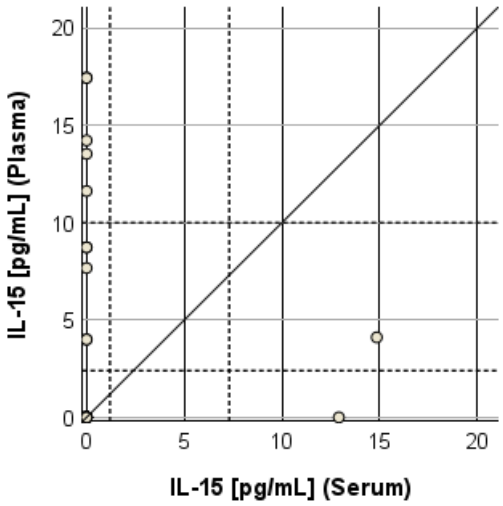
Note. The calculations were based on a 2-way mixed-effects, single-rater, absolute agreement model. Both serum and plasma samples were collected within 24 hours of bronchoscopy. Serum samples were collected, processed, and stored under standardized conditions by investigators. Plasma samples were collected within routine clinical care; thus, there was a higher variability in processing and storing of samples. $N = 44$. CCL5 = cysteine-cysteine motif chemokine ligand 5; ICC = intraclass correlation coefficient.

Figure 34 *Comparison of Concentrations of Interleukin 8 in Plasma and Serum Samples*



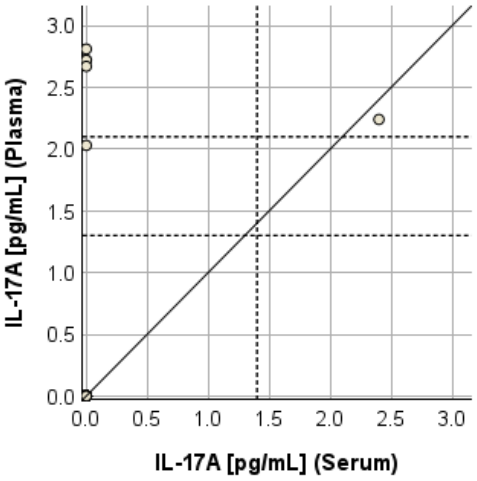
Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). The vertical dashed line represents the lower bound of the calibrated measurement range for serum levels (i.e., x = 2.0 pg/mL). The horizontal dashed line represents the lower bound of the calibrated measurement range for plasma levels (i.e., y = 2.0 pg/mL). Levels that fell below these thresholds were assigned a value of 0.0 pg/mL. The scatter plot on the right is identical to the lower concentration range of the scatter plot on the left; the scale has been changed to present the lower concentration range in a meaningful way, at the expense of charting outliers. *N* = 44. IL = interleukin.

Figure 35 *Comparison of Concentrations of IL-15 in Plasma and Serum Samples*



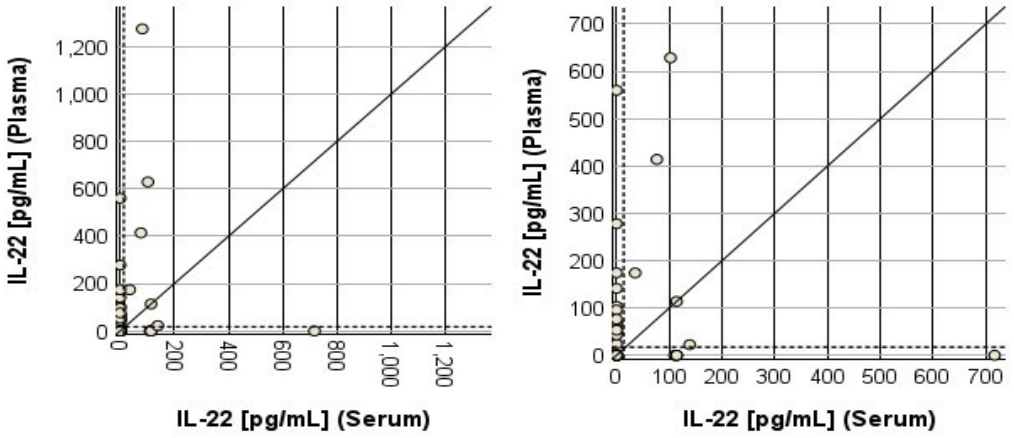
Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). The vertical dashed lines represent the lower bounds of the calibrated measurement range for serum levels (i.e., x = 1.2 pg/mL and x = 7.3 pg/mL). The horizontal dashed lines represent the lower bounds of the calibrated measurement range for plasma levels (i.e., y = 2.4 pg/mL and y = 10.0 pg/mL). The disparity in calibrated measurement range bounds can be explained by different dates of sample analysis. Levels that fell below these thresholds were assigned a value of 0.0 pg/mL. *N* = 44. IL = interleukin.

Figure 36 *Comparison of Concentrations of IL-17A in Plasma and Serum Samples*



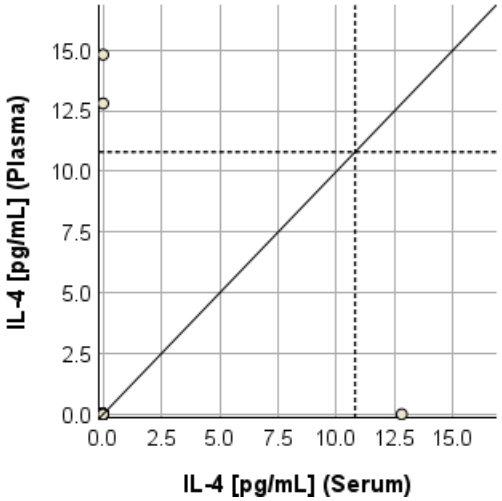
Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). The vertical dashed line represents the lower bound of the calibrated measurement range for serum levels (i.e., x = 1.4 pg/mL). The horizontal dashed lines represent the lower bounds of the calibrated measurement range for plasma levels (i.e., y = 1.3 pg/mL and y = 2.1 pg/mL). The disparity in calibrated measurement range bounds can be explained by different dates of sample analysis. Levels that fell below these thresholds were assigned a value of 0.0 pg/mL. *N* = 44. IL = interleukin.

Figure 37 *Comparison of Concentrations of IL-22 in Plasma and Serum Samples*



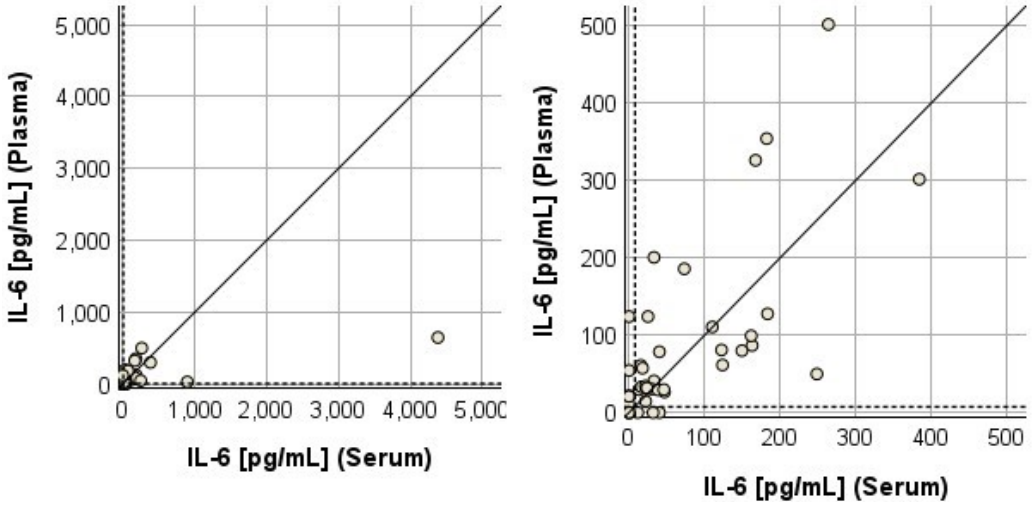
Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). The vertical dashed line represents the lower bound of the calibrated measurement range for serum levels (i.e., x = 13.5 pg/mL). The horizontal dashed line represents the lower bound of the calibrated measurement range for plasma levels (i.e., y = 17.3 pg/mL). Levels that fell below these thresholds were assigned a value of 0.0 pg/mL. The scatter plot on the right is identical to the lower concentration range of the scatter plot on the left; the scale has been changed to present the lower concentration range in a meaningful way, at the expense of charting outliers. *N* = 44. IL = interleukin.

Figure 38 *Comparison of Concentrations of Interleukin 4 in Plasma and Serum Samples*



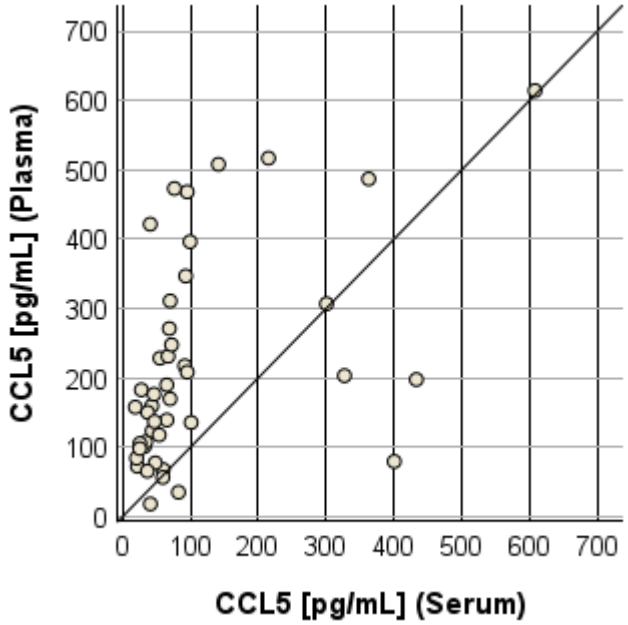
Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). The vertical dashed line represents the lower bound of the calibrated measurement range for serum levels (i.e., x = 10.8 pg/mL). The horizontal dashed line represents the lower bound of the calibrated measurement range for plasma levels (i.e., y = 10.8 pg/mL). Levels that fell below these thresholds were assigned a value of 0.0 pg/mL. *N* = 44. IL = interleukin.

Figure 39 *Comparison of Concentrations of Interleukin 6 in Plasma and Serum Samples*



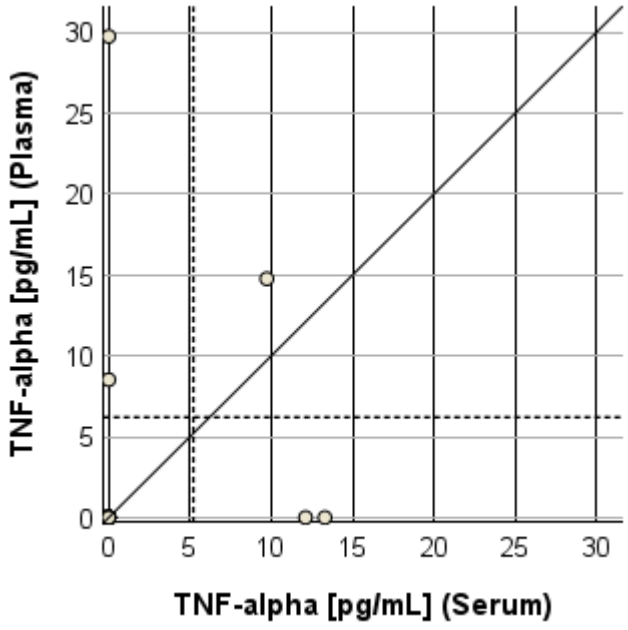
Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). The vertical dashed line represents the lower bound of the calibrated measurement range for serum levels (i.e., x = 7.9 pg/mL). The horizontal dashed line represents the lower bound of the calibrated measurement range for plasma levels (i.e., y = 7.4 pg/mL). Levels that fell below these thresholds were assigned a value of 0.0 pg/mL. The scatter plot on the right is identical to the lower concentration range of the scatter plot on the left; the scale has been changed to present the lower concentration range in a meaningful way, at the expense of charting outliers. *N* = 44. IL = interleukin.

Figure 40 *Comparison of Concentrations of CCL5 in Plasma and Serum Samples*



Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). $N = 44$. CCL5 = cysteine-cysteine motif chemokine ligand 5.

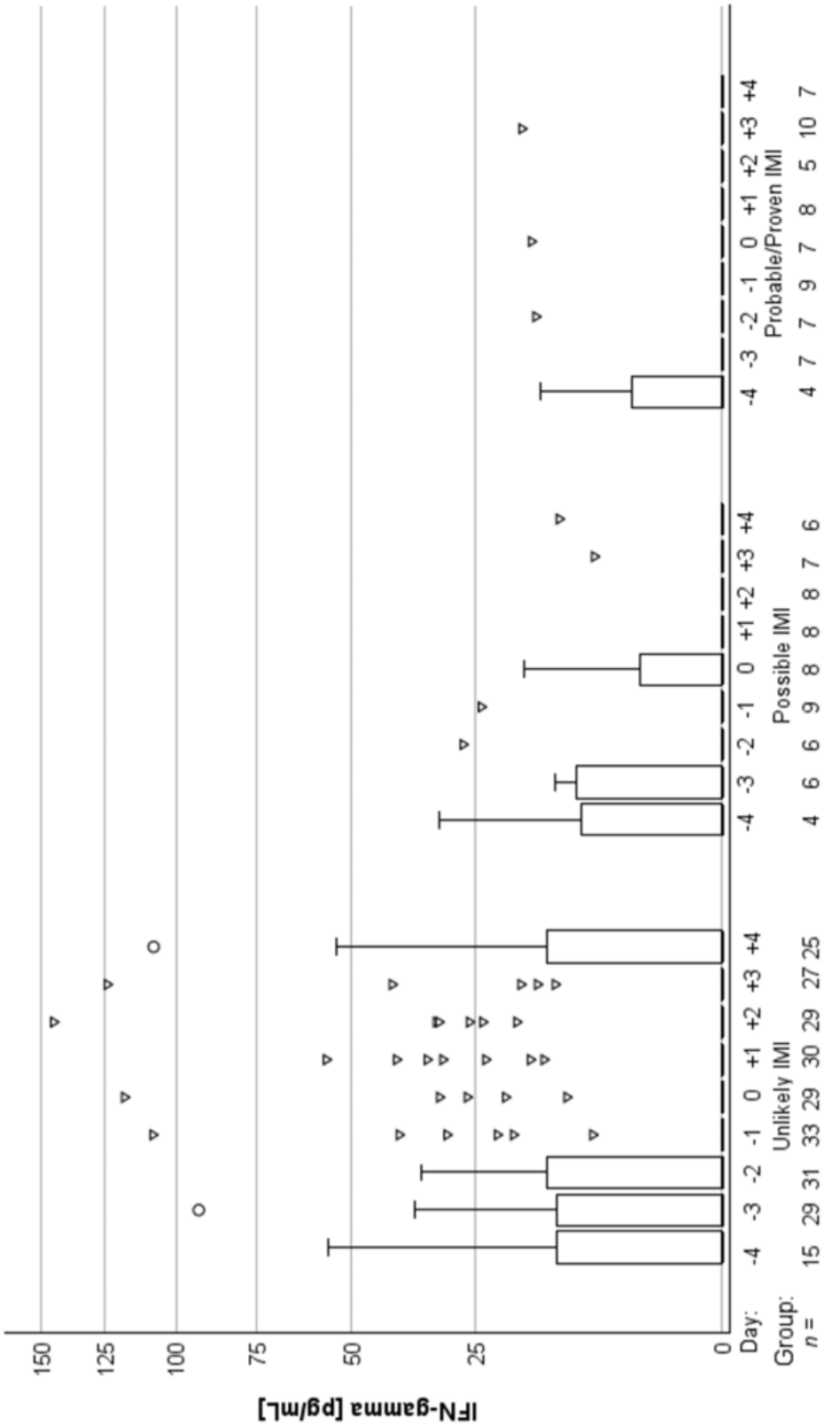
Figure 41 *Comparison of Concentrations of TNF-α in Plasma and Serum Samples*



Note. The plasma samples were collected within the clinical routine (i.e., not standardized), whereas the serum samples were collected by the investigators (i.e., standardized). Both sample types were collected within 24 hours of bronchoscopy. Each dot represents an individual case. The diagonal continuous line is the reference line (i.e., y = x). The vertical dashed line represents the lower bound of the calibrated measurement range for serum levels (i.e., x = 5.2 pg/mL). The horizontal dashed line represents the lower bound of the calibrated measurement range for plasma levels (i.e., y = 6.2 pg/mL). Levels that fell below these thresholds were assigned a value of 0.0 pg/mL. $N = 44$. TNF = tumor necrosis factor.

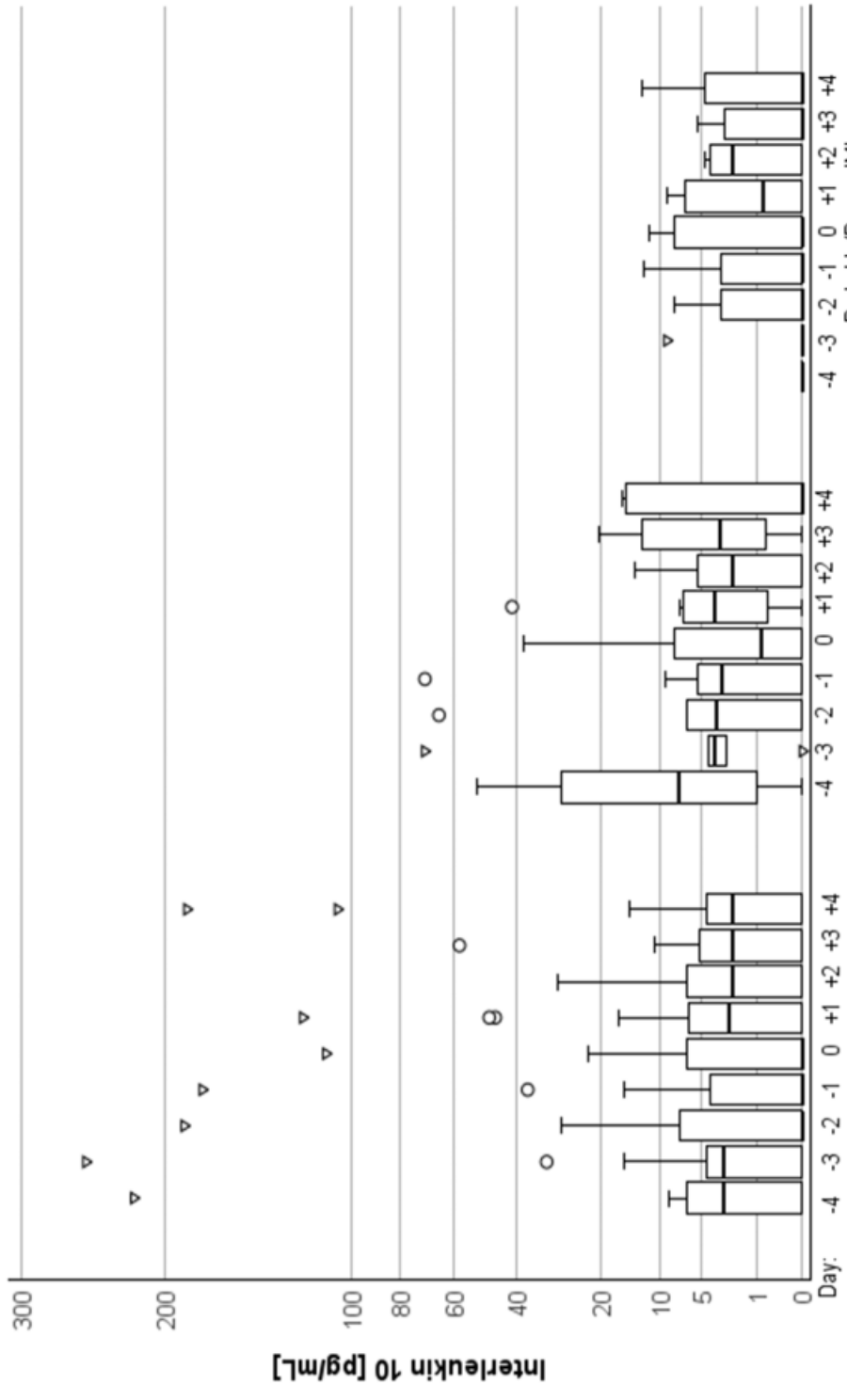
With respect to IFN-γ levels in plasma, the unlikely IMI group exhibited the highest individual levels (see Figure 42). However, medians for each group and for each day were 0.00 pg/mL. Levels of IL-10 were found to be lower in the probable/proven IMI group—most of the medians were 0.00 pg/mL—compared to the other groups (see Figure 43); the highest median value was observed in the possible IMI group on day –4 (*Mdn* = 8.11 pg/mL, IQR = 41.0). Similarly, sIL-2R levels also tended to be lower in probable/proven IMI cases in comparison to the other groups. The highest median value of sIL-2R was observed in the possible IMI group on day –3, while the lowest median value was recorded in the probable/proven IMI group on day –2; *Mdn* = 64,558.52 pg/mL, IQR = 60,363.6; and *Mdn* = 10,917.29 pg/mL, IQR = 37,655.1; respectively (see Figure 44). However, it should be noted that these summaries encompassed all samples obtained. The development of biomarker concentrations in cases where paired samples were available is demonstrated for IFN-γ in Table 31 and Figure 45, for IL-10 in Table 32 and Figure 46, and for sIL-2R in Table 33 and Figure 47. For these three biomarkers, no noticeable changes in concentrations were observed over time. In fact, just a sole biomarker revealed significant differences in the longitudinal plasma concentration analysis within only one group: Only CCL5 concentrations in the possible IMI group ($n = 7$) were noticeably higher on day +3 than on day –1, Wilcoxon's signed-rank test $z = –2.37$, $p = .016$, $r = .90$; however, the underlying measured values were defined as questionable in terms of reliability.

Figure 42 *Interferon γ Concentrations in Plasma Before and After Bronchoscopy*



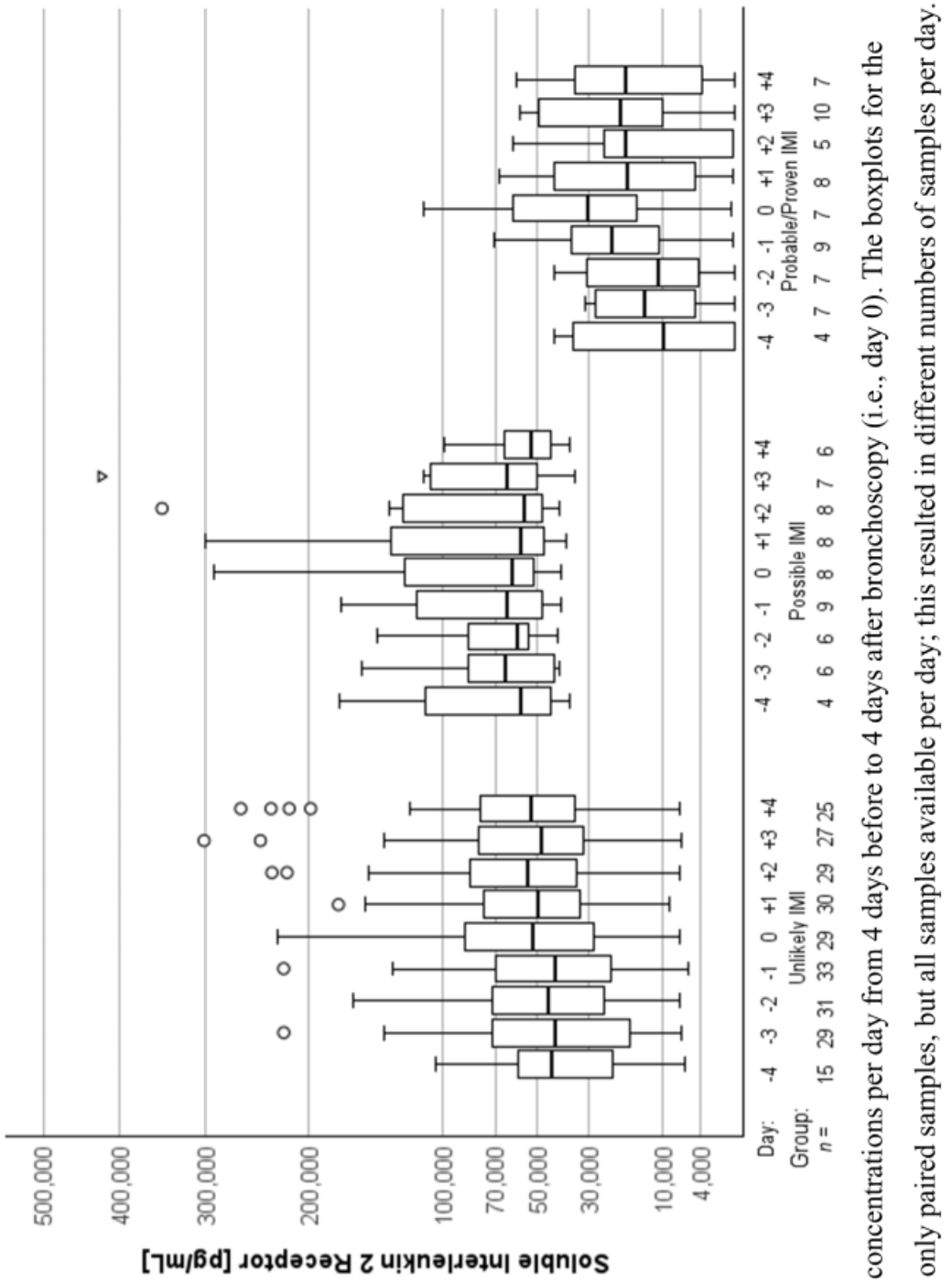
Note. Boxplots of plasma concentrations per day from 4 days before to 4 days after bronchoscopy (i.e., day 0). The boxplots for the different days include not only paired samples, but all samples available per day; this resulted in different numbers of samples per day. To allow outliers and extreme values to be displayed, the y-axis was transformed with an exponent of 0.5. $N = 59$. ○ = outliers; ▽ = extreme values; IMI = invasive mold infection; IFN = interferon.

Figure 43 *Interleukin 10 Concentrations in Plasma Before and After Bronchoscopy*



Note. Boxplots of plasma concentrations per day from 4 days before to 4 days after bronchoscopy (i.e., day 0). The boxplots for the different days include not only paired samples, but all samples available per day; this resulted in different numbers of samples per day. To allow outliers and extreme values to be displayed, the y-axis was transformed with an exponent of 0.5. $N = 59$. ○ = outliers; ▽ = extreme values; IMI = invasive mold infection.

Figure 44 *Concentrations of sIL-2R in Plasma Before and After Bronchoscopy*



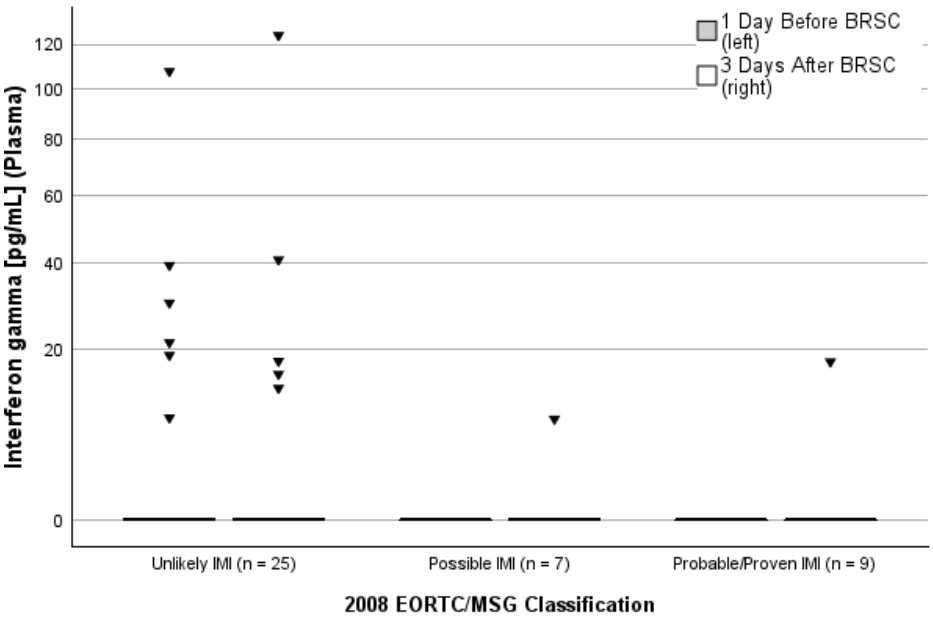
Note. Boxplots of plasma concentrations per day from 4 days before to 4 days after bronchoscopy (i.e., day 0). The boxplots for the different days include not only paired samples, but all samples available per day; this resulted in different numbers of samples per day. To allow outliers and extreme values to be displayed, the y-axis was transformed with an exponent of 0.5. $N = 59$. ○ = outliers; ▽ = extreme values; IMI = invasive mold infection; sIL-2R = soluble interleukin 2 receptor.

Table 31 *Changes in IFN-γ Concentrations During 4 Days*

Day/period	*Mdn*	95% CI	Q1	Q3	IQR	Min	Max	*p* (*r*)
Unlikely invasive mold infections (*n* = 25)								
–1d	0.00		0.0	0.0	0.0	0.0	107.8	.866
+3d	0.00		0.0	0.0	0.0	0.0	124.1	(.03)
–1d to +3d	0.00	[0.00, 21.91]	0.0	0.0	0.0	–39.3	21.9	
Possible invasive mold infections (*n* = 7)								
–1d	0.00		0.0	0.0	0.0	0.0	0.0	.317
+3d	0.00		0.0	0.0	0.0	0.0	8.7	(.38)
–1d to +3d	0.00		0.0	0.0	0.0	0.0	8.7	
Probable/proven invasive mold infections (*n* = 9)								
–1d	0.00		0.0	0.0	0.0	0.0	0.0	.317
+3d	0.00		0.0	0.0	0.0	0.0	17.7	(.33)
–1d to +3d	0.00		0.0	0.0	0.0	0.0	17.7	

Note. Descriptive statistics of interferon γ concentrations in pg/mL 1 day before bronchoscopy (–1d) and 3 days after bronchoscopy (+3d), as well as descriptive statistics of differences in concentrations between both days (–1d to +3d). The analysis was restricted to cases for which plasma samples were available at both time points ($N = 41$). The differences in concentrations between –1d and +3d within a group were tested using Wilcoxon's signed-rank test, and subsequently the effect size *r* was calculated. The significance level was $\alpha = .05$. No correction was made for multiple testing. The corresponding 95% confidence intervals (CI) listed refer to the medians; however, calculation was not possible in some cases. IFN = interferon; IQR = interquartile range; Max = maximum; Min = minimum; Q1 = 1st quartile/25th percentile; Q3 =3rd quartile/75th percentile.

Figure 45 *Changes in IFN-γ Concentrations in Plasma During 4 Days*



Note. The y-axis has been transformed with an exponent of 0.5 to display extreme values (▼). BRSC = bronchoscopy; EORTC = European Organization for Research and Treatment of Cancer/ Invasive Fungal Infections Cooperative Group; IFN = interferon; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

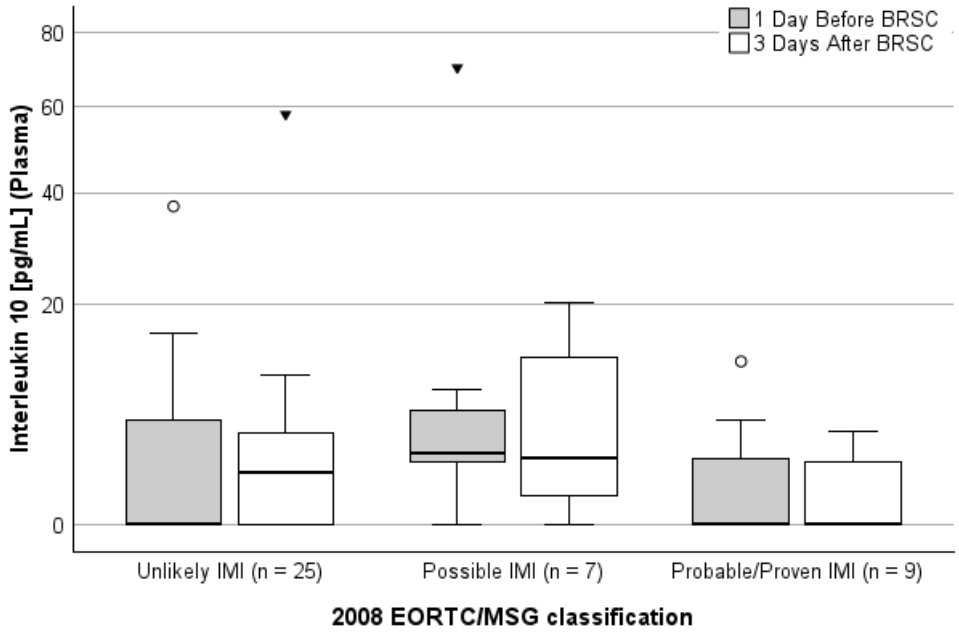
Table 32 *Changes in IL-10 Concentrations During 4 Days*

Day/period	*Mdn*	95% confidence interval	Q1	Q3	IQR	Min	Max	*p* (*r*)
Unlikely invasive mold infections (*n* = 25)								
–1d	0.00		0.0	6.3	6.3	0.0	37.2	.865
+3d	2.36	[0.00, 4.63]	0.0	5.1	5.1	0.0	57.9	(.03)
–1d to +3d	0.00	[0.00, 2.37]	–0.6	0.6	1.2	–34.4	57.9	
Possible invasive mold infections (*n* = 7)								
–1d	3.69	[2.89, 70.15]	2.9	9.3	6.4	0.0	70.2	.600
+3d	3.37	[0.00, 20.30]	0.0	18.6	18.6	0.0	20.3	(.20)
–1d to +3d	–0.15	[–3.13, 11.03]	–3.1	2.7	5.8	–51.5	11.0	
Probable/proven invasive mold infections (*n* = 9)								
–1d	0.00		0.0	3.2	3.2	0.0	12.3	.465
+3d	0.00		0.0	3.0	3.0	0.0	5.3	(.24)
–1d to +3d	0.00	[0.00, 2.25]	–0.2	0.0	0.2	–7.0	2.3	

Note. Descriptive statistics of interleukin 10 concentrations in pg/mL 1 day before bronchoscopy (–1d) and 3 days after bronchoscopy (+3d), as well as descriptive statistics of

differences in concentrations between both days(–1d to +3d). The analysis was restricted to cases for which plasma sample were available at both time points ($N = 41$). The differences in concentrations between –1d and +3d within a group were tested using Wilcoxon's signed-rank test, and subsequently the effect size r was calculated. The significance level was $\alpha = .05$. No correction was made for multiple testing. The corresponding 95% confidence intervals (CI) listed refer to the medians; however, calculation was not possible in some cases.
IL = interleukin; IQR = interquartile range; Max = maximum; Min = minimum; Q1 = 1st quartile/25th percentile; Q3 =3rd quartile/75th percentile.

Figure 46 *Changes in IL-10 Concentrations in Plasma During 4 Days*



Note. Classification of invasive mold infections (IMI) followed the 2008 revised European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC)/National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) consensus criteria. The y-axis has been transformed with an exponent of 0.5 to clearly display the boxplots, outliers (○), and extreme values (▼). BRSC = bronchoscopy; EORTC = European Organization for Research and Treatment of Cancer/ Invasive Fungal Infections Cooperative Group; IL = interleukin; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

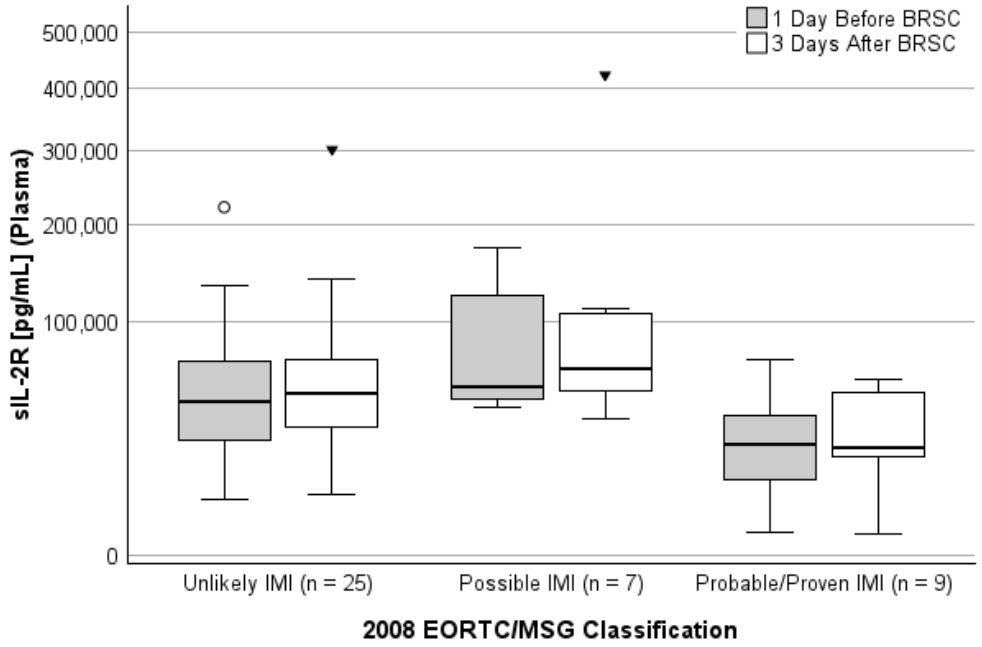
Table 33 *Changes in sIL-2R Concentrations During 4 Days*

Day/period	*Mdn*	95% CI	Q1	Q3	IQR	Min	Max	*p* (*r*)
Unlikely invasive mold infections (*n* = 25)								
–1d	43,468.79	[28,301.17; 60,094.05]	24,539.5	69,536.4	44,996.9	5,793.6	222,428.0	.427
+3d	48,207.14	[37,059.48; 66,359.27]	30,635.0	70,498.1	39,863.1	6,797.8	302,029.5	(.16)
–1d to +3d	–916.14	[–3,177.08; 13,173.73]	–4,073.6	15,371.1	19,444.7	–43,170.1	79,601.5	
Possible invasive mold infections (*n* = 7)								
–1d	52,160.31	[41,691.68; 172,933.18]	41,691.7	131,554.7	89,863.1	40,124.4	172,933.2	.612
+3d	64,388.65	[43,377.18; 422,635.38]	43,377.2	111,403.4	68,026.2	34,757.1	422,635.4	(.19)
–1d to +3d	1,685.50	[–5,367.28; 249,702.20]	–5,367.3	12,228.3	17,595.6	–27,206.9	249,702.2	
Probable/proven invasive mold infections (*n* = 9)								
–1d	22,619.80	[10,779.16; 69,290.71]	10,779.2	36,069.8	25,290.6	963.0	70,312.5	.767
+3d	21,192.84	[18,281.29; 57,160.20]	18,281.3	49,315.4	31,034.1	894.9	57,344.9	(.10)
–1d to +3d	–95.58	[–4,506.98; 10,413.68]	–4,507.0	5,093.9	9,600.9	–12,967.6	13,245.6	

Note. Descriptive statistics of soluble interleukin 2 receptor concentrations in pg/mL 1 day before bronchoscopy (–1d) and 3 days after bronchoscopy (+3d), as well as descriptive statistics of differences in concentrations between both days (–1d to +3d). The analysis was restricted to cases for which plasma sample were available at both time points ($N = 41$). The differences in concentrations between –1d and +3d within a group were tested using Wilcoxon's signed-rank test, and subsequently the effect size *r* was calculated. The significance level was $\alpha = .05$. No correction was made for multiple testing. The corresponding 95% confidence intervals (CI) listed refer to the medians. IQR = interquartile

range; Max = maximum; Min = minimum; Q1 = 1st quartile/25th percentile; Q3 =3rd quartile/75th percentile; sIL-2R = soluble interleukin 2 receptor.

Figure 47 *Changes in sIL-2R Concentrations in Plasma During 4 Days*



Note. The y-axis has been transformed with an exponent of 0.5 to clearly display the boxplots, outliers (○), and extreme values (▼). BRSC = bronchoscopy; EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; sIL-2R = soluble interleukin 2 receptor; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

Aspergillus-Specific Diagnostics

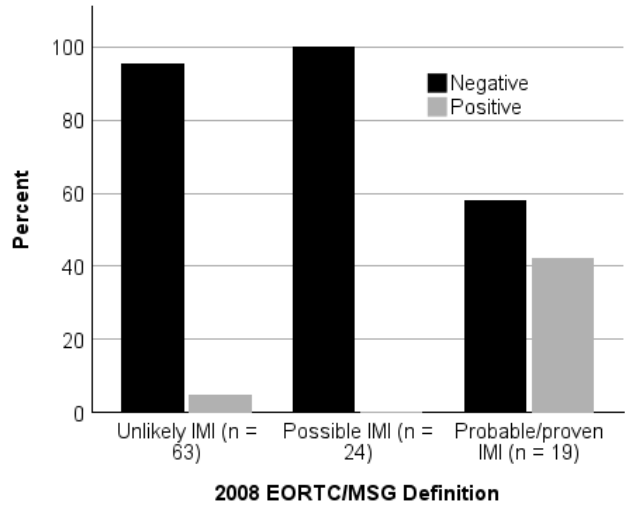
The results of the AspLFDs performed with BALF are presented in Table 34 and Figure 48. Probable/proven IMI cases exhibited a relatively high percentage of positive AspLFD test results (8/19, 42%). Conversely, no positive AspLFD test result was observed within the possible IMI group. The chi-square test was not informative as the expected counts of positive AspLFD results were less than five within the possible and probable/proven IMI groups.

Table 34 *AspLFD Results With Bronchoalveolar Lavage Fluid*

Test result	2008 EORTC/MSG definition			Total
	Unlikely IMI (n = 63)	Possible IMI (n = 24)	Probable/proven IMI (n = 19)	(*N* = 106)
negative	60	24	11	95
	95%	100%	58%	90%
positive	3 $_{a}$	0 $_{a}$	8 $_{b}$	11
	5%	0%	42%	10%

Note. Frequencies of *Aspergillus* Lateral-Flow Device (AspLFD) test results by probability of invasive mold infection (IMI). Subsets of the positive test result row sharing a common subscript were not statistically different at α = .05 (pairwise Fisher's exact test, with Bonferroni correction applied). EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

Figure 48 *AspLFD Results with Bronchoalveolar Lavage Fluid*



Note. Relative frequencies of *Aspergillus* Lateral-Flow Device (AspLFD) test results by probability of invasive mold infection (IMI). *N* = 106. EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

A subsequent pairwise Fisher's exact tests with Bonferroni corrections demonstrated that the probable/proven IMI group exhibited a clearly higher frequency of positive AspLFD test results in comparison to the unlikely or possible IMI group, adjusted $p < .001$, odds ratio (OR) = 14.55, $n = 82$; and adjusted $p = .002$, $n = 43$ (OR was not computable mathematically); respectively. The analysis could not find a significant difference between the unlikely and possible IMI groups, adjusted $p = 1.0$, OR = 0.00, $n = 87$. When evaluating results regarding IPAs, 5% (3/63) of AspLFDs in the unlikely IPA group, 0% (0/32) of AspLFDs in the possible IPA group, and 73% (8/11) of AspLFDs in the probable IPA group were positive. This resulted in a high specificity of 97% with the ability to highly increase the post-test probability of an IMI (LR+ = 12.21), but also a low sensitivity of 42% (see Table 35; for diagnosing IPA: 73% [8/11] sensitivity, 97% [92/95] specificity). There was only poor agreement with galactomannan in BALF, cavities in chest CT scans, or galactomannan in serum, $\kappa = .56$, $p < .001$; $\kappa = .43$, $p < .001$; and $\kappa = .22$, $p = .008$; respectively (see Table 24).

Five *Aspergillus* PCR results with BALF were missing (5/106, 5%; i.e., four unlikely IMI and one possible IMI cases): In two cases, PCR failed due to technical reasons, in another two cases volumes of BALF residues were insufficient, and one sample was lost during transit to the collaborating laboratory in Mannheim, Germany. The majority of positive results belonged to the probable/proven IMI group (3/19, 16%; see Table 36 and Figure 49). A single positive *Aspergillus* PCR result was identified in each of the unlikely and possible IMI groups. The chi-square test did not provide information as the expected counts of positive PCR results were less than five in all groups. A pairwise Fisher's exact test with Bonferroni correction revealed no statistically significant differences between the unlikely and probable/proven, possible and probable/proven, and unlikely and possible IMI groups, adjusted $p = .129$, OR = 10.88, $n = 78$; adjusted $p = .939$, OR = 4.13, $n = 42$; and adjusted $p = 1.0$, OR = 2.64, $n = 82$; respectively. When evaluating results regarding IPAs, 2% (1/59) of *Aspergillus* PCRs in the unlikely IPA group, 3% (1/31) of *Aspergillus* PCRs in the possible IPA group, and 27% (3/11) of *Aspergillus* PCRs in the probable IPA group were positive.

Table 35 *Diagnostic Test Performance for the AspLFD and the* Aspergillus *PCR With BALF*

Diagnostic test characteristics		
		95% confidence interval
Aspergillus Lateral-Flow Device [a]		
Youden's index	.39	
Sensitivity	42%	[22%, 64%]
Specificity	97%	[91%, 99%]
Positive predictive value	73%	[44%, 92%]
Negative predictive value	88%	[81%, 94%]
Diagnostic odds ratio	20.36	[4.69, 88.41]
Positive likelihood ratio	12.21	[3.57, 41.80]
Negative likelihood ratio	0.61	[0.41, 0.88]
Aspergillus polymerase chain reaction [b]		
Youden's index	.14	
Sensitivity	16%	[4%, 36%]
Specificity	98%	[93%, 100%]
Positive predictive value	60%	[20%, 92%]
Negative predictive value	83%	[75%, 90%]
Diagnostic odds ratio	7.50	[1.16, 48.56]
Positive likelihood ratio	6.47	[1.16, 36.09]
Negative likelihood ratio	0.86 [†]	[0.71, 1.05]

Note. Values pertain to the differentiation of probable/proven invasive mold infections (IMI) from unlikely/possible IMIs. AspLFD = *Aspergillus* Lateral-Flow Device; BALF = bronchoalveolar lavage fluid; PCR = polymerase chain reaction.

[a] $N = 106$ (87 unlikely/possible IMIs and 19 probable/proven IMIs). [b] $N = 101$ (82 unlikely/possible IMIs and 19 probable/proven IMIs).

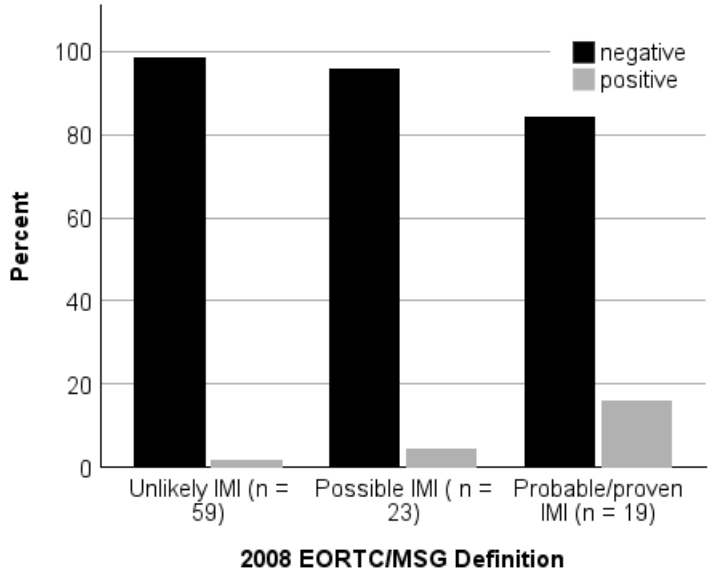
[†] Not significant, as the 95% confidence interval included the value 1.00.

Table 36 Aspergillus *PCR Results With Bronchoalveolar Lavage Fluid*

Test result	2008 EORTC/MSG definition			Total
	Unlikely IMI (n = 59)	Possible IMI (n = 23)	Probable/proven IMI (n = 19)	(*N* = 101)
negative	58	22	16	96
	98%	96%	84%	95%
positive	1 $_{a}$	1 $_{a}$	3 $_{a}$	5
	2%	4%	16%	5%

Note. Subsets of the positive test result row sharing a common subscript were not statistically different at α = .05 (pairwise Fisher's exact test, Bonferroni correction was applied). EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; IMI = invasive mold infection; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

Figure 49 *Results of* Aspergillus *PCR with Bronchoalveolar Lavage Fluid*



Note. Relative frequencies of *Aspergillus* polymerase chain reaction (PCR) test results with bronchoalveolar lavage fluid. *N* = 101. EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; IMI = invasive mold infection; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

Compared to the AspLFD, the *Aspergillus* PCR demonstrated a similar specificity of 98% and exhibited the potential to approve a diagnosis of IMI (LR+ = 6.47), but also exhibited a very low sensitivity of 16% (for diagnosing IPA: 27% [3/11] sensitivity, 98% [88/90] specificity; see Table 35). The 95% CI of the negative LR included the value of 1.00, LR– = 0.86, 95% CI [0.71, 1.05]. In comparison with the AspLFD, the *Aspergillus* PCR test demonstrated poorer agreements with the galactomannan assay using BALF and with cavitary lesions observed on chest CT scans (see Table 24).

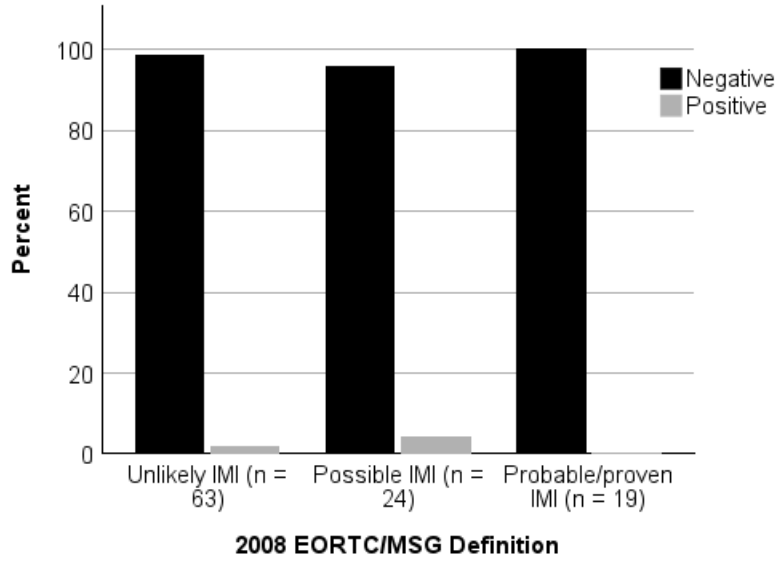
The AspLFD with serum and the *Aspergillus* PCR with whole blood exhibited no diagnostic potential, as there were no true-positive test results. A mere 2% (2/106) of all cases exhibited a positive AspLFD test with serum samples, with one case each in the unlikely and possible IMI group (see Table 37 and Figure 50). The chi-square test was not informative because the expected counts of positive AspLFD test results were less than five within all groups. There was no demonstrable difference in frequency of positive AspLFD test results between the unlikely and probable/proven, the possible and probable/proven, and unlikely and possible IMI group, and pairwise Fisher's exact tests (adjusted by Bonferroni correction) demonstrated adjusted $p = 1.0$, OR = 0.00, $n = 82$; $p = 1.0$, OR = 0.00, $n = 43$; $p = 1.0$, OR = 2.70, $n = 87$. When evaluating results regarding IPAs, 2% (1/63) of AspLFDs in the unlikely IPA group, 3% (1/32) of AspLFDs in the possible IPA group, and 0% (0/11) of AspLFDs in the probable IPA group were positive. All whole blood samples that were tested using the *Aspergillus* PCR returned negative results. Missing PCR results occurred in 19% (12/106): In six cases PCR failed due to technical reasons, in another five cases volumes of whole blood samples were insufficient, and one sample was lost during transit to the collaborating laboratory in Mannheim, Germany. Cases with missing data belonged to the unlikely (8/63, 13%), the possible (2/24, 8%), and the probable/proven (2/19, 11%) IMI group.

Table 37 *Frequencies of AspLFD Results With Serum*

Test result	2008 EORTC/MSG definition			Total
	Unlikely IMI (n = 63)	Possible IMI (n = 24)	Probable/proven IMI (n = 19)	(N = 106)
Negative	62	23	19	104
	98%	96%	100%	98%
Positive	1 $_a$	1 $_a$	0 $_a$	2
	2%	4%	0%	2%

Note. Subsets of the positive test results row sharing a common subscript were not statistically different at α = .05 (pairwise Fisher's exact test, Bonferroni correction was applied). AspLFD = *Aspergillus* Lateral-Flow Device; EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; IMI = invasive mold infection; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

Figure 50 *Frequencies of AspLFD Results With Serum*



Note. N = 106. AspLFD = *Aspergillus* Lateral-Flow Device; EORTC = European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; IMI = invasive mold infection; MSG = National Institute of Allergy and Infectious Diseases Mycoses Study Group.

Associations Between Biomarker Levels and Mortality

The analysis of BALF samples revealed that patients who deceased within 90 days of bronchoscopy exhibited slightly elevated levels of IL-6 in their BALF in comparison to those patients who survived 90 days, MWU $U = 688.00$, $p = .003$, $r = .29$ (see Table 38 and Figure 51). Similarly, slightly elevated concentrations of IL-6 and also IL-8 were observed in serum, MWU $U = 777.50$, $p = .017$, $r = .23$; and $U = 753.50$, $p = .012$, $r = .25$; respectively (see Table 39, Figure 52, and Figure 53). However, the 95% CIs of the medians for IL-6 in serum overlapped, suggesting that the MWU indicated a different probability distribution rather than a clear difference in the central tendency between the groups. Within the group of probable/proven IMIs, however, IL-6 and IL-8 were not associated with mortality within 90 days. Instead, slightly higher IL-22 concentrations were observed in the BALF (*Mdn* = 141.42 pg/mL, 95% CI [101.60, 281.25], IQR [57.2, 257.4]) of non-deceased patients with probable/proven IMI ($n = 12$) compared to the BALF of deceased patients with probable/proven IMI (*Mdn* = 42.42 pg/mL, 95% CI [42.42, 76.49], IQR [0.0, 42.4], $n = 7$), MWU $U = 10.50$, $p = .005$, $r = .26$ (see Figure 54). No noticeable differences in IL-8 concentrations in BALF in connection with 90-day mortality were observed (see Figure 55), nor were any differences observed for the other biomarkers (see Table 38 and Table 39; and Figure 56 for IL-15, Figure 57 for sIL-2R, Figure 58 for IL-22, Figure 59 for CCL5, and Figure 60 for IL-10).

Table 38 *Descriptive Analysis of Biomarker Concentrations in BALF by 90-Day Mortality*

Statistics	90-Day mortality		*p*
	Not deceased (*n* = 76)	Deceased (*n* = 29)	(*r*)
	Interferon γ		
Median	0.00	0.00	.486
95% confidence interval			(.07)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	251.6	8.8	
	Interleukin 10		
Median	0.00	0.00	.967
95% confidence interval			(.00)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	21.8	4.1	
	Interleukin 15		
Median	0.00	4.11	.736
95% confidence interval		[4.11, 8.44]	(.03)
Percentile 25	0.0	0.0	
Percentile 75	7.0	4.1	
Interquartile range	7.0	4.1	
Minimum	0.0	0.0	
Maximum	62.1	35.6	

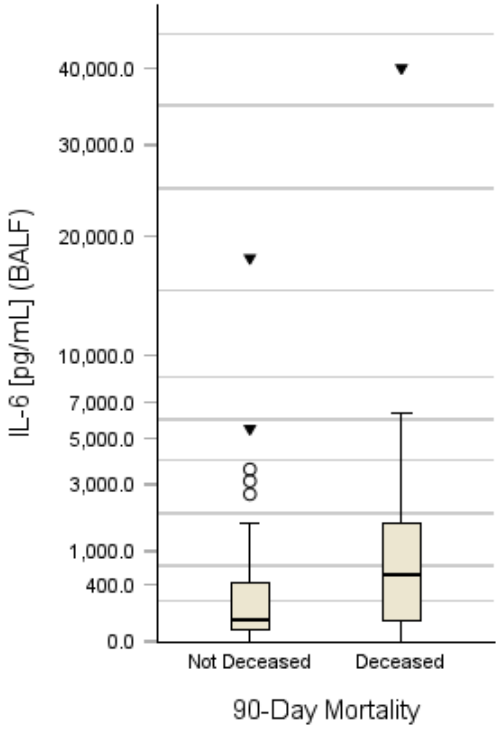
Statistics	90-Day mortality		*p*
	Not deceased (*n* = 76)	Deceased (*n* = 29)	(*r*)
	Interleukin 17A		
Median	0.00	0.00	.550
95% confidence interval			(.06)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	31.3	12.4	
	Soluble interleukin 2 receptor		
Median	235.86	308.74	.477
95% confidence interval	[129.08, 308.74]	[121.20, 498.65]	(.07)
Percentile 25	38.0	96.7	
Percentile 75	526.4	765.1	
Interquartile range	488.4	668.4	
Minimum	0.0	0.0	
Maximum	40,771.4	11,150.7	
	Interleukin 22		
Median	56.47	42.42	.135
95% confidence interval	[42.42, 101.60]	[42.42, 101.60]	(.15)
Percentile 25	0.0	0.0	
Percentile 75	141.4	101.6	
Interquartile range	141.4	101.6	
Minimum	0.0	0.0	
Maximum	1,217.1	877.4	

Statistics	90-Day mortality		*p*
	Not deceased (*n* = 76)	Deceased (*n* = 29)	(*r*)
	Interleukin 4		
Median	0.00	0.00	.683
95% confidence interval			(.04)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	28.1	11.9	
	Interleukin 6		
Median	53.72	532.52	.003
95% confidence interval	[32.52, 143.95]	[198.35; 1,215.35]	(.29)
Percentile 25	16.4	56.3	
Percentile 75	415.0	1,727.6	
Interquartile range	398.6	1,671.3	
Minimum	0.0	0.0	
Maximum	17,899.4	40,000.0	
	Interleukin 8		
Median	595.60	1,009.33	.102
95% confidence interval	[460.77, 833.25]	[607.81; 2,066.74]	(.16)
Percentile 25	296.5	453.6	
Percentile 75	1,438.9	2,079.4	
Interquartile range	1,142.4	1,625.8	
Minimum	58.4	78.7	
Maximum	6,754.1	5,672.7	

Statistics	90-Day mortality		*p*
	Not deceased (*n* = 76)	Deceased (*n* = 29)	(*r*)
Cysteine-Cysteine motif chemokine ligand 5			
Median	9.53	6.30	.271
95% confidence interval	[5.04, 12.99]	[3.68, 11.22]	(.11)
Percentile 25	3.4	2.6	
Percentile 75	26.3	16.9	
Interquartile range	22.9	14.3	
Minimum	0.0	0.0	
Maximum	258.7	105.0	
Tumor necrosis factor α			
Median	0.00	0.00	.182
95% confidence interval			(.13)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	19.4	22.8	

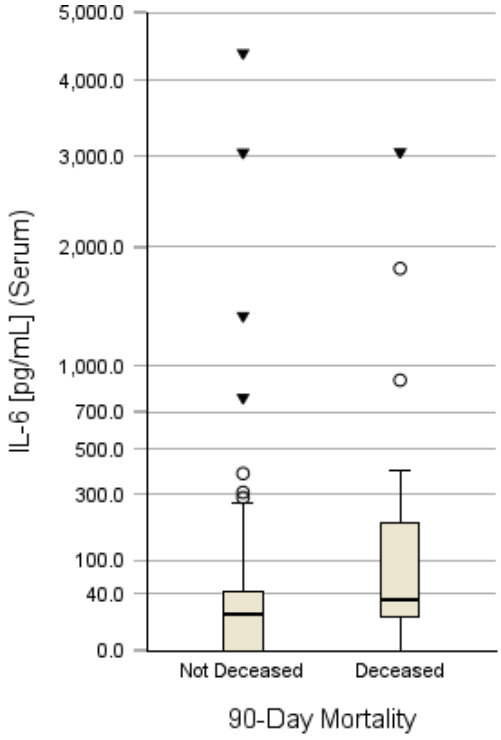
Note. The concentrations of biomarkers are given in pg/mL. The 95% confidence intervals listed refer to the medians; however, calculation was not possible in some cases. The differences in probability distributions were tested using Mann-Whitney *U*-test, and effect size *r* was calculated. The level of significance was α = .05. No adjustment was made to address multiple testing. *N* = 105. BALF = bronchoalveolar lavage fluid.

Figure 51 *Interleukin 6 Levels in BALF by 90-Day Mortality*



Note. The y-axis was transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). *N* = 105 (76 patients did not decease, 29 patients deceased within 90 days of bronchoscopy).
BALF = bronchoalveolar lavage fluid;
IL = interleukin.

Figure 52 *Interleukin 6 Levels in Serum by 90-Day Mortality*



Note. The y-axis was transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). *N* = 105 (76 patients did not decease, 29 patients deceased within 90 days of bronchoscopy). IL = interleukin.

Table 39 *Descriptive Analysis of Biomarker Concentrations in Serum by 90-Day Mortality*

Statistics	90-Day mortality		*p*
	Not deceased (*n* = 76)	Deceased (*n* = 29)	(*r*)
	Interferon γ		
Median	0.00	0.00	.416
95% confidence interval			(.08)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	105.8	36.4	
	Interleukin 10		
Median	0.00	0.00	.846
95% confidence interval			(.02)
Percentile 25	0.0	0.0	
Percentile 75	3.8	3.3	
Interquartile range	3.8	3.3	
Minimum	0.0	0.0	
Maximum	220.2	83.0	
	Interleukin 15		
Median	0.00	0.00	.523
95% confidence interval			(.06)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	14.9	14.9	

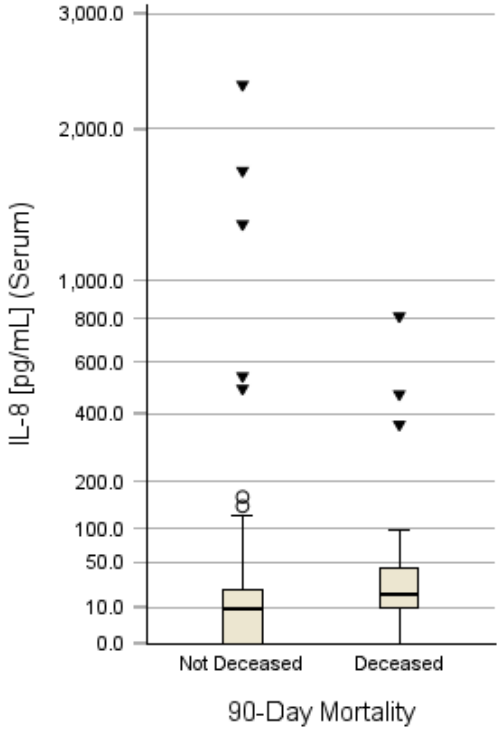
Statistics	90-Day mortality		*p*
	Not deceased (*n* = 76)	Deceased (*n* = 29)	(*r*)
	Interleukin 17A		
Median	0.00	0.00	.486
95% confidence interval			(.07)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	3.7	2.4	
	Soluble interleukin 2 receptor		
Median	36,978.31	42,761.70	.519
95% confidence interval	[30,881.49; 49,543.37]	[30,275.33; 80,193.38]	(.06)
Percentile 25	18,127.0	26,822.1	
Percentile 75	69,277.8	88,213.3	
Interquartile range	51,150.8	61,391.2	
Minimum	1,156.4	490.8	
Maximum	318,362.5	123,522.3	
	Interleukin 22		
Median	0.00	0.00	.505
95% confidence interval			(.07)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	716.6	110.1	

Statistics	90-Day mortality		*p*
	Not deceased (*n* = 76)	Deceased (*n* = 29)	(*r*)
	Interleukin 4		
Median	0.00	0.00	.537
95% confidence interval			(.06)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	12.8	0.0	
	Interleukin 6		
Median	16.54	31.83	.017
95% confidence interval	[0.00, 25.82]	[16.46, 183.61]	(.23)
Percentile 25	0.0	13.5	
Percentile 75	43.2	200.4	
Interquartile range	43.2	186.9	
Minimum	0.0	0.0	
Maximum	4,384.0	3,049.4	
	Interleukin 8		
Median	9.09	18.69	.012
95% confidence interval	[4.98, 13.56]	[13.81, 38.38]	(.25)
Percentile 25	0.0	9.6	
Percentile 75	22.2	42.5	
Interquartile range	22.2	32.9	
Minimum	0.0	0.0	
Maximum	2,359.2	808.4	

Statistics	90-Day mortality		*p*
	Not deceased (*n* = 76)	Deceased (*n* = 29)	(*r*)
Cysteine-Cysteine motif chemokine ligand 5			
Median	56.84	59.21	.863
95% confidence interval	[44.93, 68.81]	[40.93, 73.23]	(.02)
Percentile 25	32.3	36.9	
Percentile 75	100.2	76.3	
Interquartile range	67.9	39.4	
Minimum	2.9	0.0	
Maximum	608.2	506.8	
Tumor necrosis factor α			
Median	0.00	0.00	.804
95% confidence interval			(.02)
Percentile 25	0.0	0.0	
Percentile 75	0.0	0.0	
Interquartile range	0.0	0.0	
Minimum	0.0	0.0	
Maximum	12.1	13.3	

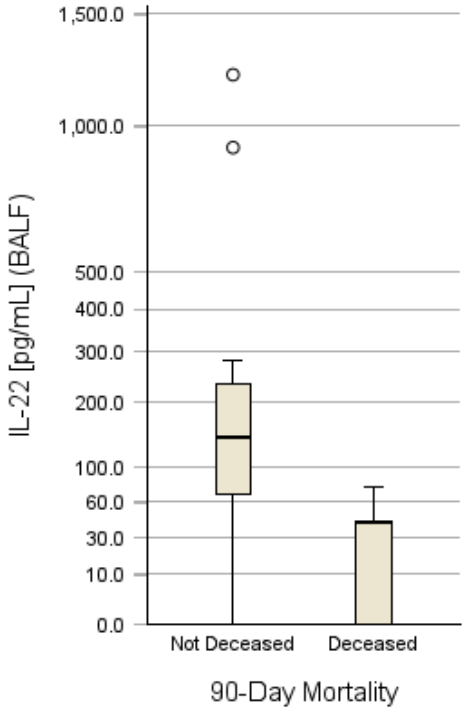
Note. The concentrations of biomarkers are given in pg/mL. The 95% confidence intervals listed refer to the medians; however, calculation was not possible in some cases. The differences in probability distributions were tested using Mann-Whitney *U*-test, and effect size *r* was subsequently calculated. The level of significance was α = .05. No adjustment was made to address multiple testing. *N* = 105.

Figure 53 *Interleukin 8 Levels in Serum by 90-Day Mortality*



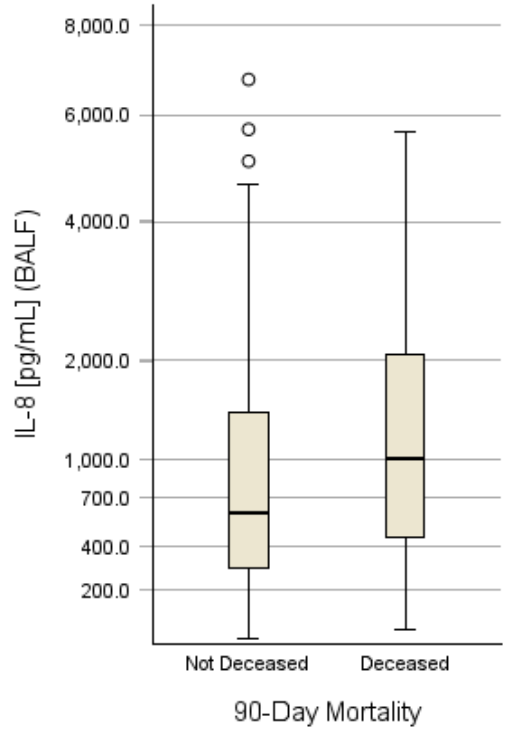
Note. The y-axis was transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). *N* = 105 (76 patients did not decease, 29 patients deceased within 90 days of bronchoscopy). IL = interleukin.

Figure 54 *IL-22 Levels in BALF of Probable/Proven IMIs by 90-Day Mortality*



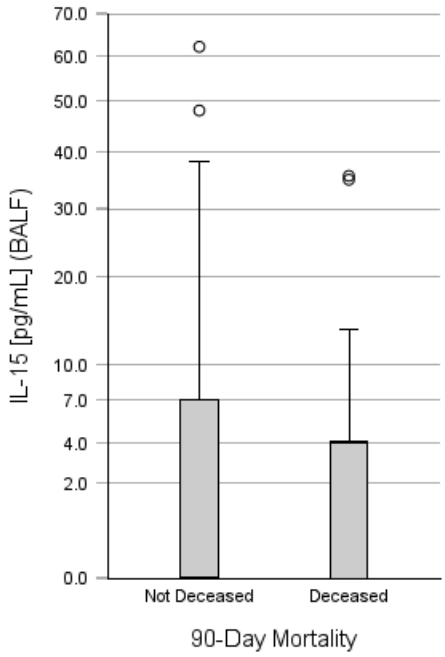
Note. The y-axis was transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots and outliers (○). *N* = 19 (12 patients survived at least 90 days after bronchoscopy and seven patients deceased within this time). BALF = bronchoalveolar lavage fluid; IL = interleukin.

Figure 55 *Interleukin 8 Levels in BALF by 90-Day Mortality*



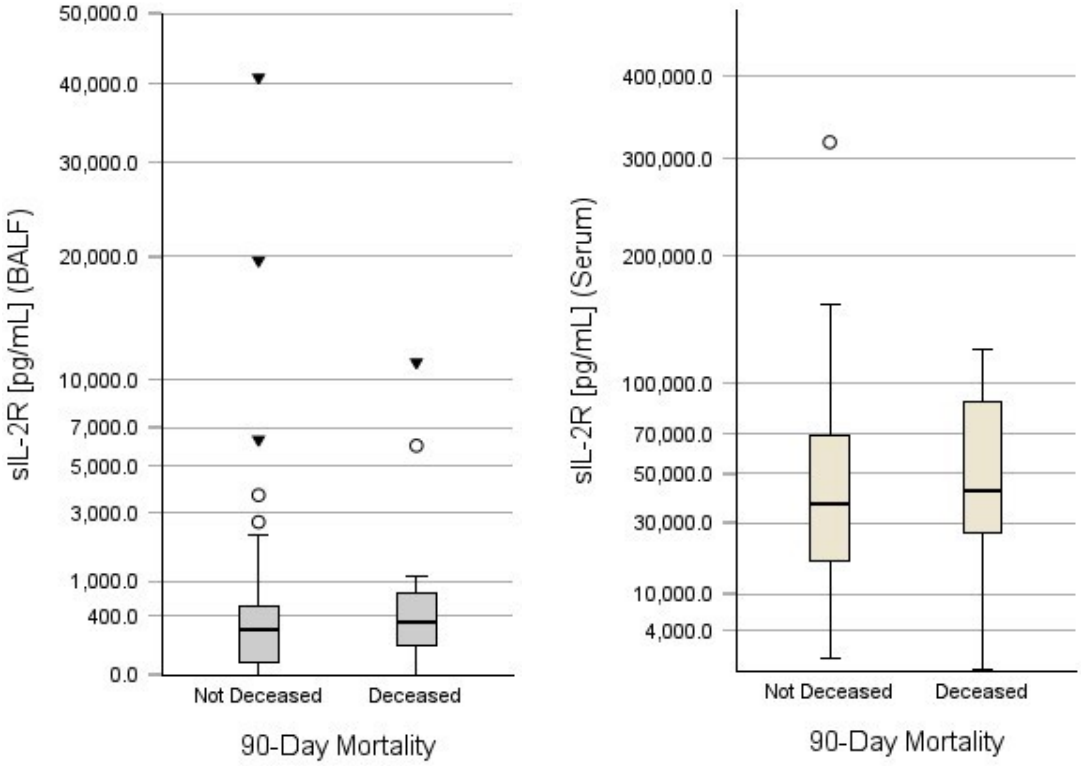
Note. The y-axis was transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots and outliers (○). *N* = 105 (76 patients did not decease, 29 patients deceased within 90 days of bronchoscopy). BALF = bronchoalveolar lavage fluid; IL = interleukin.

Figure 56 *Interleukin 15 Levels in BALF by 90-Day Mortality*



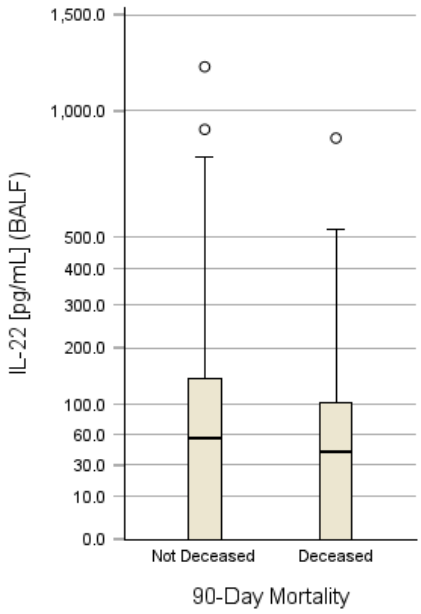
Note. The y-axis was transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots and outliers (○). *N* = 105 (76 patients did not decease, 29 patients deceased within 90 days of bronchoscopy). BALF = bronchoalveolar lavage fluid; IL = interleukin.

Figure 57 *Soluble Interleukin 2 Receptor Levels by 90-Day Mortality*



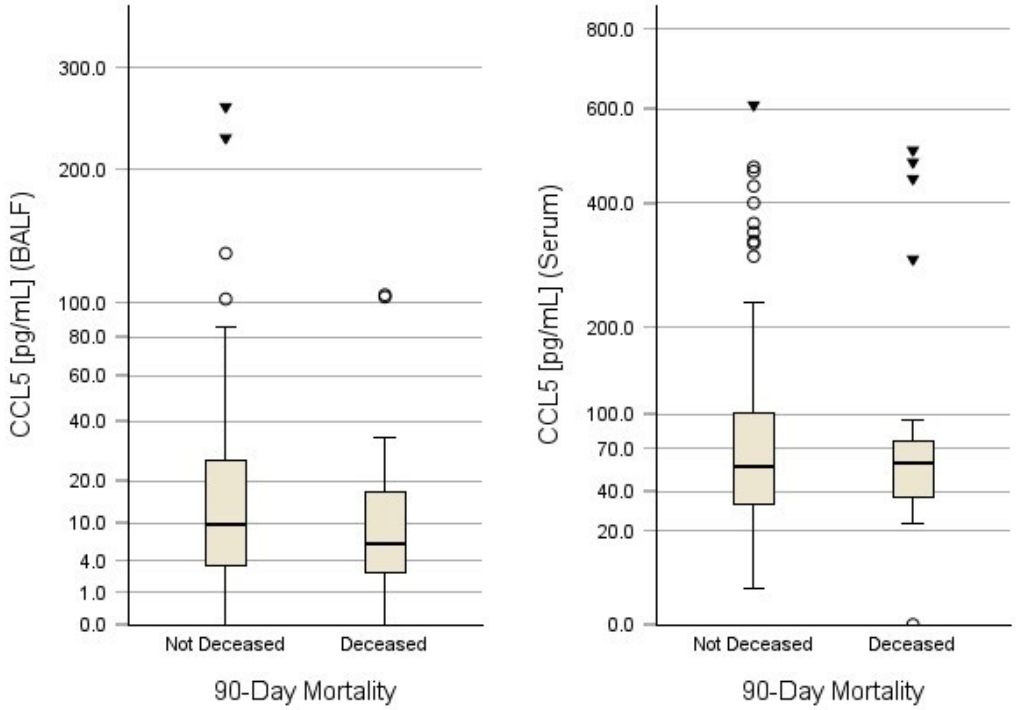
Note. The y-axes were transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). *N* = 105 (76 patients did not decease, 29 patients deceased within 90 days of bronchoscopy). BALF = bronchoalveolar lavage fluid; sIL-2R = soluble interleukin 2 receptor.

Figure 58 *IL-22 Levels in BALF of all Cases by 90-Day Mortality*



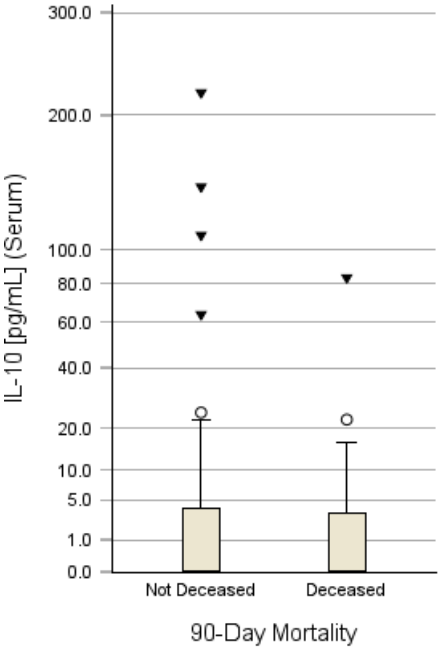
Note. The y-axis was transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots and outliers (○). *N* = 105 (76 patients did not decease, 29 patients deceased within 90 days of bronchoscopy).
BALF = bronchoalveolar lavage fluid; IL = interleukin.

Figure 59 *Cysteine-Cysteine Motif Chemokine Ligand 5 Levels by 90-Day Mortality*



Note. The y-axes were transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). *N* = 105 (76 patients did not decease, 29 patients deceased within 90 days of bronchoscopy). BALF = bronchoalveolar lavage fluid; CCL5 = cysteine-cysteine motif chemokine ligand 5.

Figure 60 *Interleukin 10 Levels in Serum by 90-Day Mortality*



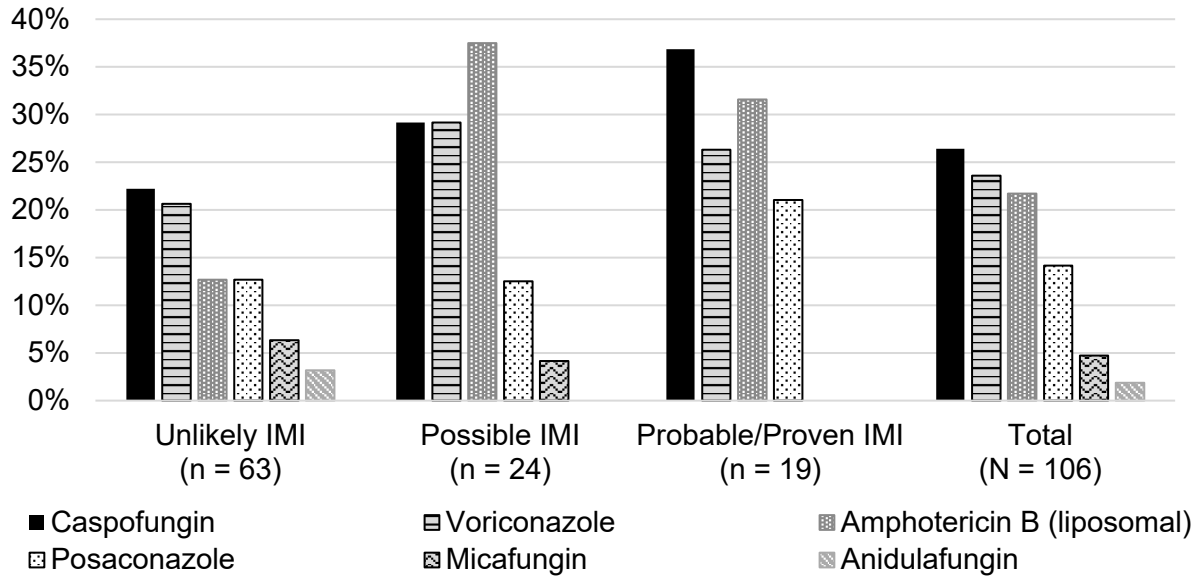
Note. The y-axis was transformed with an exponent of 0.5 to ensure optimal visibility of the boxplots, outliers (○), and extreme values (▼). *N* = 105 (76 patients did not decease, 29 patients deceased within 90 days of bronchoscopy). IL = interleukin.

Mold-Active Antifungal Therapy

In 10% (11/106) of cases, two antifungal agents were administered concurrently on the day of bronchoscopy (i.e., in 5% [3/63] of unlikely, 13% [3/24] of possible, and 26% [5/19] of probable/proven IMI cases). In one case, this occurred because agents were switched on the day of bronchoscopy (i.e., caspofungin to voriconazole). The remaining combinations included voriconazole plus micafungin (*n* = 1), voriconazole plus liposomal amphotericin B (*n* = 3), posaconazole plus liposomal amphotericin B (*n* = 1), micafungin plus liposomal amphotericin B (*n* = 1), caspofungin plus posaconazole (*n* = 3), and caspofungin plus liposomal amphotericin B (*n* = 1). On the day of bronchoscopy, the most prevalent agents administered were caspofungin, voriconazole, and liposomal amphotericin B, which were administered to 26% (28/106), 24% (25/106), and 22% (23/106) of patients, respectively (see Figure 61). Within the possible IMI group, liposomal amphotericin B was the most frequently prescribed antifungal agent (i.e., in 38% [9/24]). Within the probable/proven IMI group, only 32% (6/19) of patients received liposomal amphotericin B, whereas 37% (7/19) received caspofungin. The probable IPA cases received caspofungin or liposomal amphotericin B in 34% (4/11) each, and only 18% (2/11) of cases received voriconazole. Unlikely IMI cases were most times treated with caspofungin or voriconazole (i.e., in 22% (14/63) and 21% (13/63) of cases, respectively).

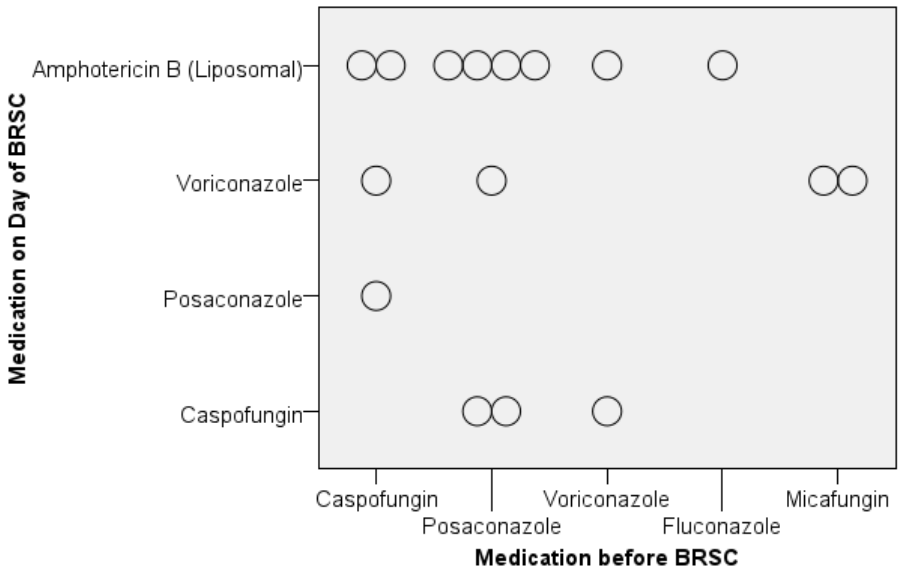
Prior to initiation of further diagnostics via bronchoscopy, antifungal medication had been changed in 16% (14/87) of cases with prescribed mold active antifungal therapies (i.e., in 15% [7/46] of unlikely, in 8% [2/24] of possible, and in 29% [5/17] of probable/proven IMI cases). Most times, posaconazole was changed to liposomal amphotericin B (see Figure 62). Results of fungal resistance testing were available for the two probable IMI cases with cultural growth of *Scedosporium apiospermum*. The results indicated that the therapies administered (i.e., liposomal amphotericin B and caspofungin; see Table 14) were not efficacious at the time of bronchoscopy; both resistance testing results revealed minimum inhibitory concentrations of higher than 32 mg/L.

Figure 61 *Mold-Active Antifungal Medication on Day of Bronchoscopy*



Note. Ten percent of cases received two antifungal agents on day of bronchoscopy; therefore, cumulative percentages may exceed 100%. Probabilities of invasive mold infections (IMI) were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group consensus definitions. IMI = invasive mold infection.

Figure 62 *Changes in Mold-Active Antifungal Therapy Prior to Bronchoscopy*



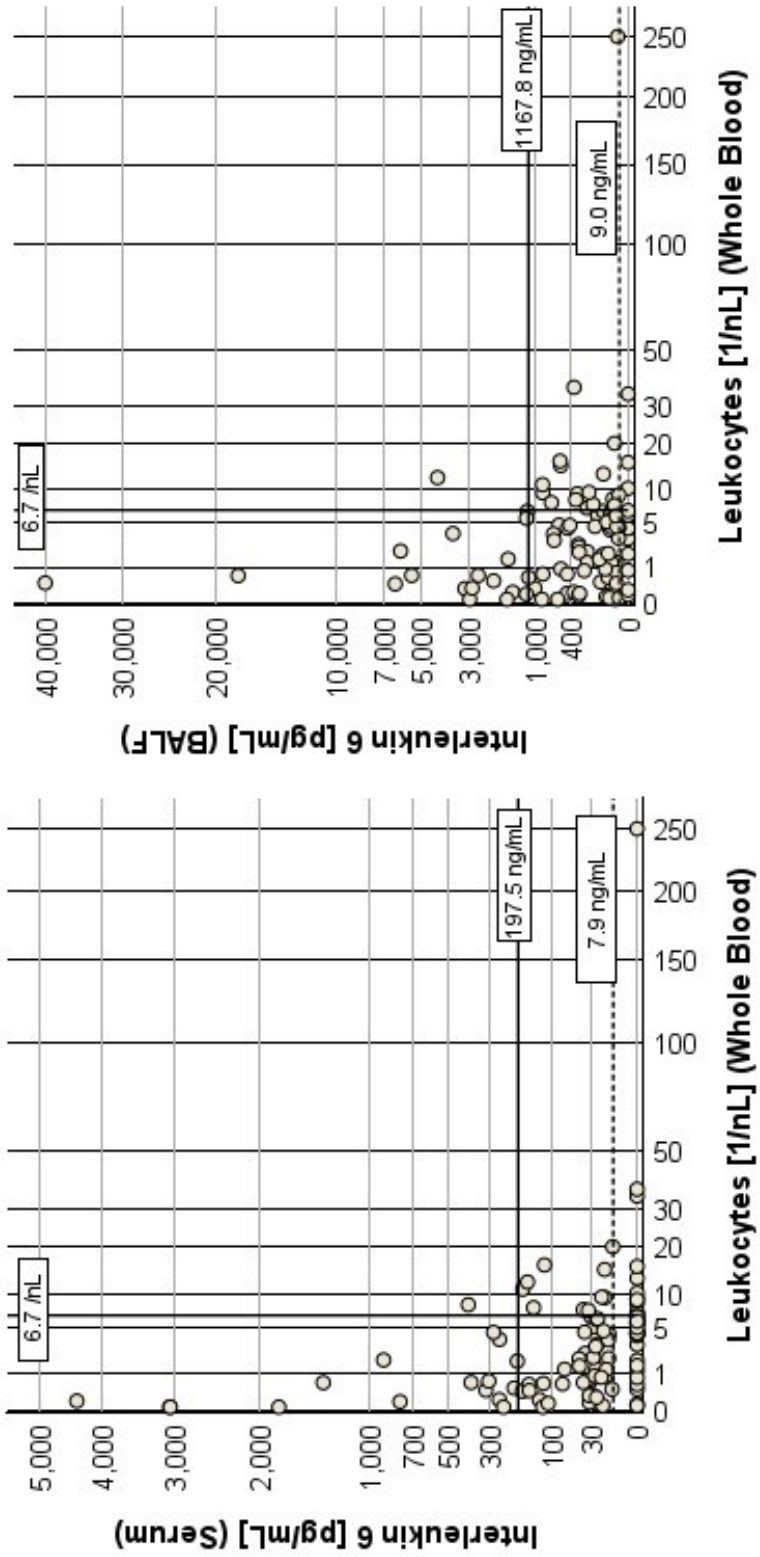
Note. Antifungal agents administered and changed before bronchoscopy (BRSC). Total count exceeds the number of cases ($N = 14$) as one case had received two agents on day of bronchoscopy, and one case had received two agents during the period before bronchoscopy.

Post-Hoc Analyses for Identifying Possible Influencing Factors

Leukocytes are a significant source of cytokines and chemokines. Leukocyte and neutrophil counts in our cohort were low (i.e., medians were in the range of leukopenia and neutropenia), at 2.55 /nL, IQR 5.7; and 0.86 /nL, IQR 3.7; respectively. Thus, possible correlations between the immunological biomarkers and cytopenia were examined. Bivariate correlation analyses revealed possible non-linear negative correlations between, on the one hand, the leukocyte count in whole blood and, on the other hand, IL-10, IL-6, and IL-8 in serum, or IL-10, IL-17A, IL-6, and TNF-α in BALF, respectively. However, the visual inspection of the respective scatter plots indicated correlations (i.e., a hyperbolic correlation) only between (a) the leukocyte count in whole blood and (b) concentrations of IL-6 in serum and BALF (see Figure 63) and IL-8 in serum (see Figure 64). The corresponding correlation coefficients were as follows: $\tau = -.34$, $p < .001$ for IL-6 in serum, $\tau = -.16$, $p = .019$ for IL-6 in BALF, and $\tau = -.39$, $p < .001$ for IL-8 in serum. The scatter plots depicting IL-10, IL-17A, TNF-α, and IL-8 concentrations in BALF did not reveal discernible distribution patterns that would suggest associations or exhibited too many values below the calibrated measurement range to be meaningfully analyzable. However, the negative correlations between leukocyte counts and IL-6 and IL-8 are more likely indicative of an association between a severe infection—or a specific type of infection—due to an impaired cellular immune defense. A direct causal relationship between low leukocyte counts and high IL-6 and IL-8 levels is not objectively plausible.

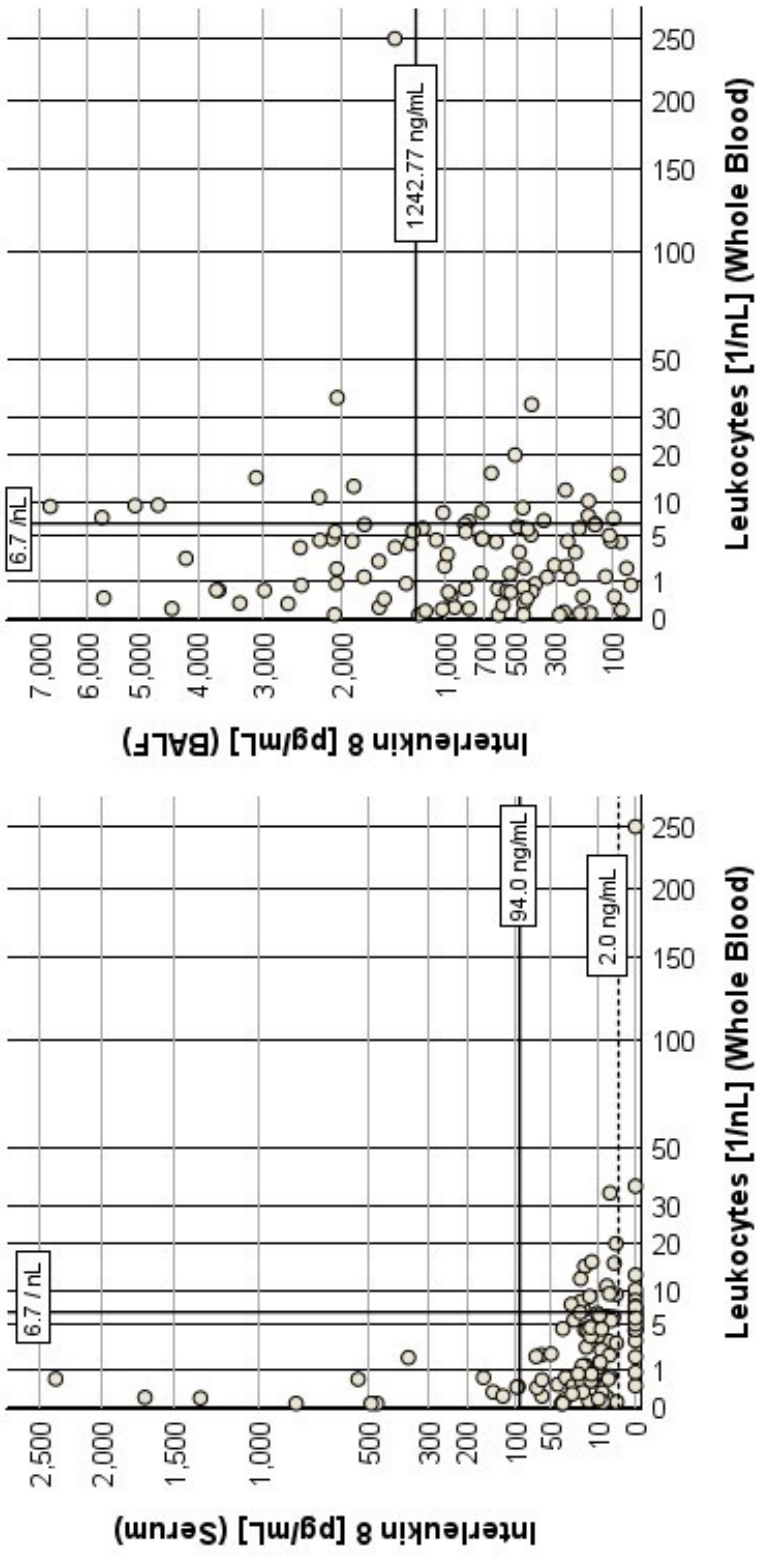
Analyses were conducted to identify associations between underlying diseases and biomarker levels for the three most prevalent underlying diseases in our study population, namely, AML, NHL, and acute lymphoblastic leukemia (ALL). Levels of IL-8 in serum (see Figure 65) and IL-6 levels in BALF (see Figure 66) were slightly elevated in cases of ALL ($n = 11$) compared to cases of other underlying diseases ($n = 95$), MWU $U = 305.50$, $p = .024$, $r = .22$; and $U = 326.00$, $p = .042$, $r = .20$; respectively. In cases of AML ($n = 47$), concentrations of CCL5 in BALF (see Figure 67) were slightly lower than in other underlying diseases ($n = 59$), Welch's t-test $t(84.510) = 2.045$, $p = .044$, $d = .37$. In cases of NHL, no strikingly different biomarker concentrations were observed in serum and BALF when compared to biomarker levels in other underlying diseases.

Figure 63 *Associations of Interleukin 6 Concentrations and Leukocyte Counts*



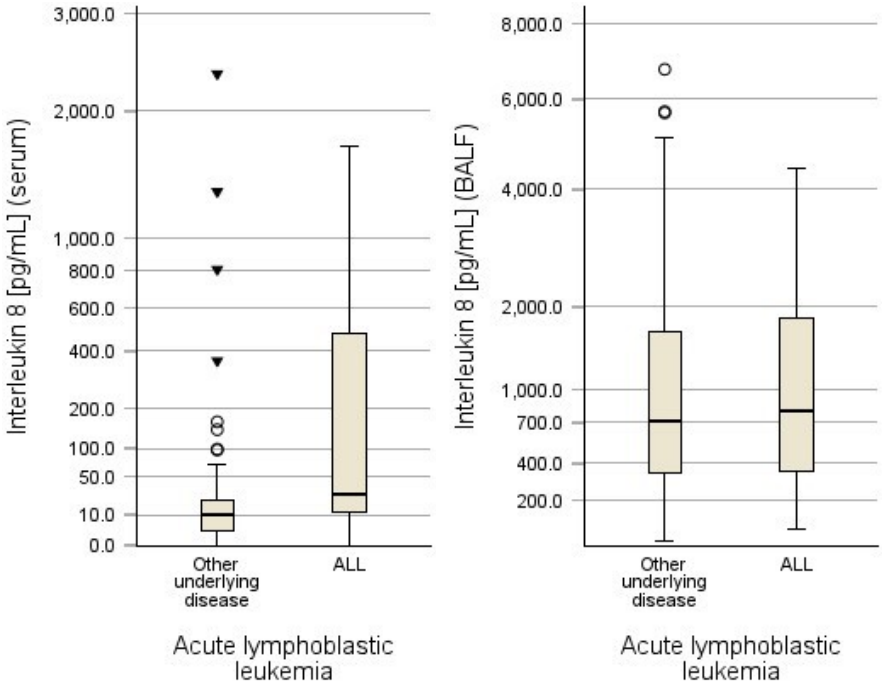
Note. The axes are transformed with an exponent of 0.5 to preserve visual interpretability despite outliers and extreme values. The vertical lines represent the mean leukocyte concentration, the horizontal continuous lines the mean interleukin (IL) 6 concentrations, the horizontal dashed lines the lower bounds of the calibrated measurement ranges of the IL-6 assay. $N = 106$. BALF = bronchoalveolar lavage fluid.

Figure 64 *Associations of Interleukin 8 Concentrations and Leukocyte Counts*



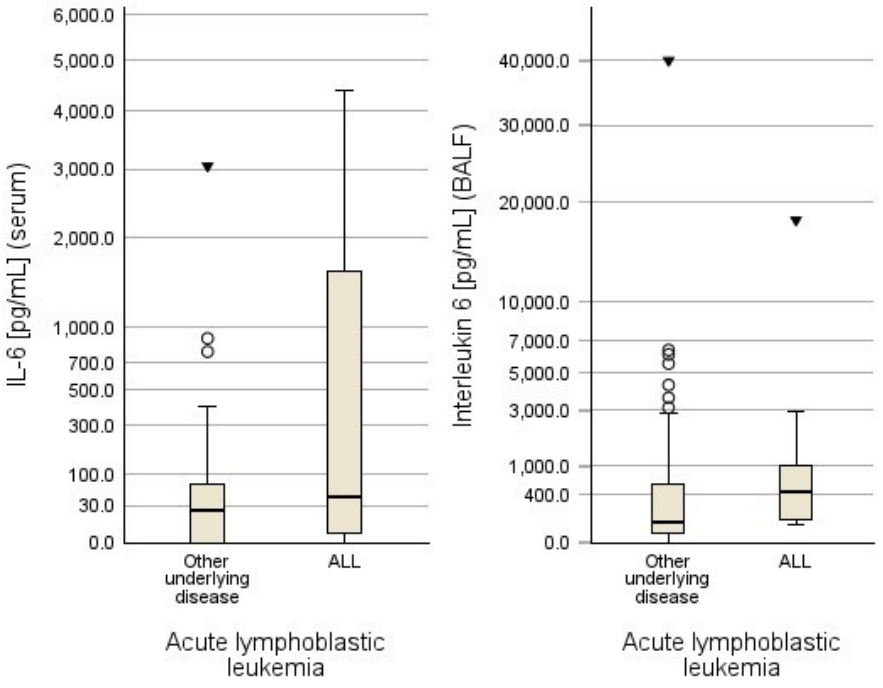
Note. The axes are transformed with an exponent of 0.5 to preserve visual interpretability despite outliers and extreme values. The vertical lines represent the mean leukocyte concentration, the horizontal continuous lines the mean interleukin (IL) 8 concentrations, the horizontal dashed line the lower bound of the calibrated measurement range of the IL-8 assay with serum samples (with bronchoalveolar lavage fluid [BALF] samples, each measurement was within the calibrated measurement range). $N = 106$.

Figure 65 *Concentrations of Interleukin 8 in Cases With Acute Lymphoblastic Leukemia*



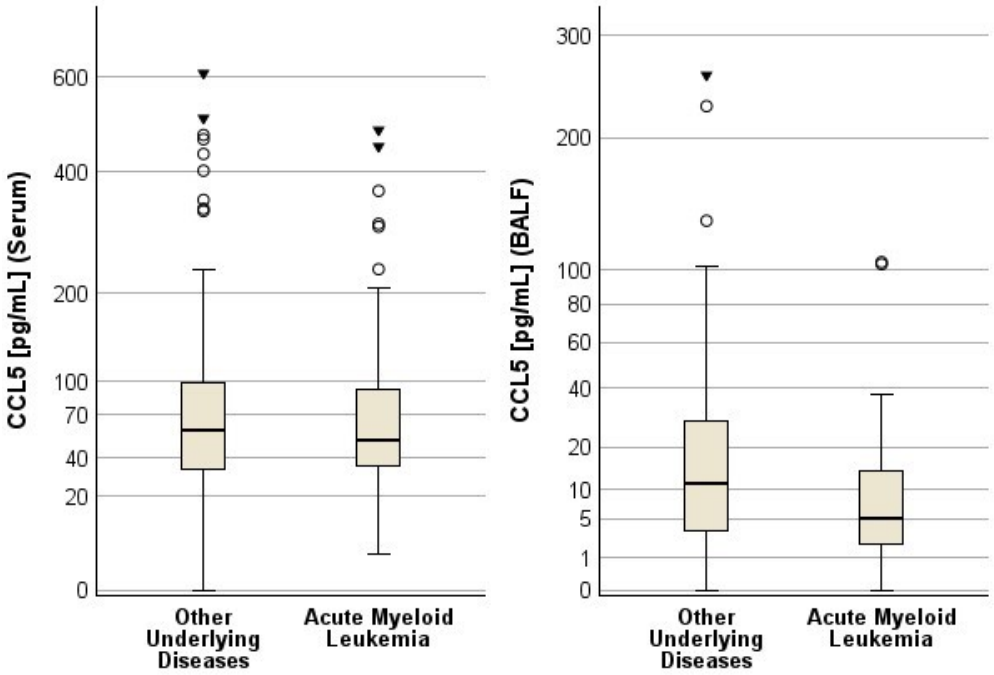
Note. $N = 106$ (95 cases with underlying diseases other than acute lymphoblastic leukemia [ALL], 11 cases with ALL as the underlying disease). The y-axes were transformed with an exponent of 0.5 to clearly display boxplots, outliers (○), and extreme values (▼). BALF = bronchoalveolar lavage fluid.

Figure 66 *Concentrations of Interleukin 6 in Cases With Acute Lymphoblastic Leukemia*



Note. $N = 106$ (95 cases with underlying diseases other than acute lymphoblastic leukemia [ALL], 11 cases with ALL as the underlying disease). The y-axes were transformed with an exponent of 0.5 to clearly display boxplots, outliers (○), and extreme values (▼). BALF = bronchoalveolar lavage fluid; IL = interleukin.

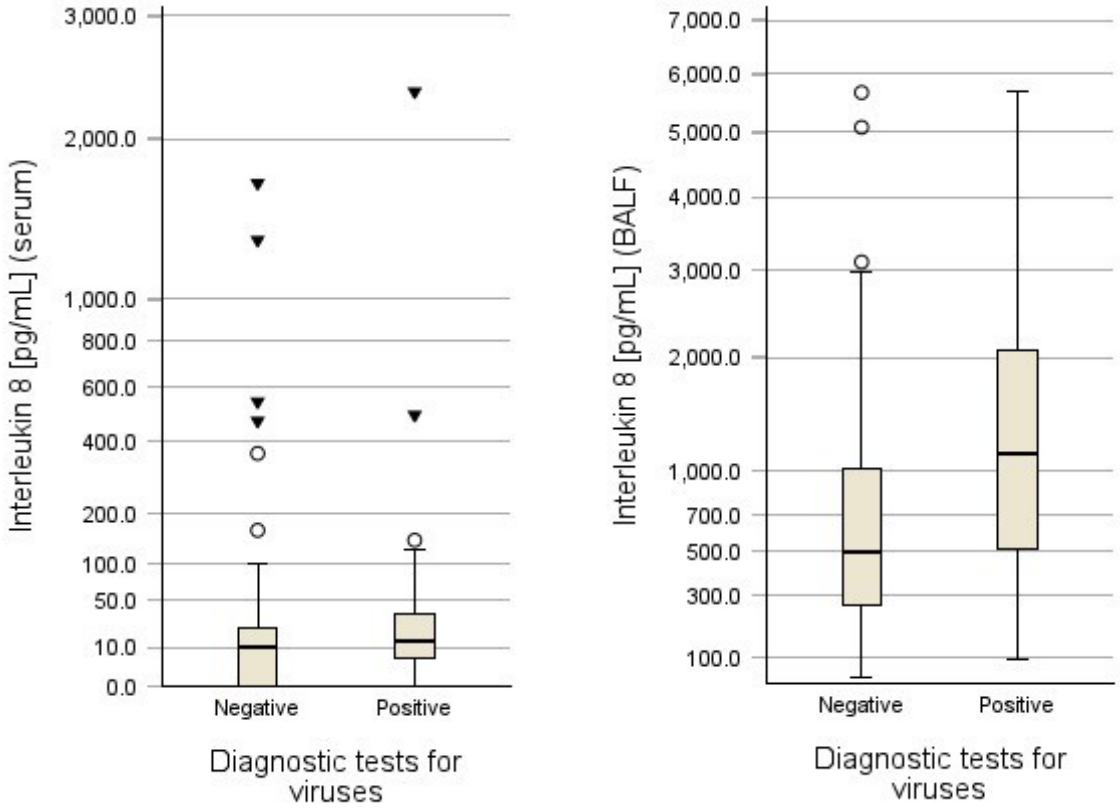
Figure 67 *Concentrations of CCL5 in Cases With Acute Myeloid Leukemia*



Note. $N = 106$ (59 cases with underlying diseases other than acute myeloid leukemia [AML], 47 cases with AML as the underlying disease). The y-axes were transformed with an exponent of 0.5 to clearly display boxplots, outliers (○), and extreme values (▼).
BALF = bronchoalveolar lavage fluid; CCL5 = cysteine-cysteine motif chemokine ligand 5.

Viral infections (i.e., 61 cases with positive virus test results within 14 days of bronchoscopy) were associated with a moderately increased level of IL-8 in the BALF compared to cases that did not exhibit positive virus test results ($n = 38$), Welch's t-test $t(63.935) = –2.392$, $p = .020$, $d = –.53$ (see Figure 68). In probable/proven IMI cases that exhibited IL-8 levels above our optimal cut-off of 877.7 pg/mL ($n = 14$), EBV was the most frequently detected virus: EBV was observed in 29% (4/14) of these cases, followed by HSV-1 and CMV in 21% (3/14) of cases each. Furthermore, influenza A, rhino/enterovirus, and parainfluenza were each observed in 7% (1/14) of cases with IL-8 concentrations above the proposed cut-off in BALF.

Figure 68 *Interleukin 8 Concentrations in Cases With Positive Virus Test Results*



Note. *N* = 99 (61 cases with positive virus test results within 14 days of bronchoscopy, 38 cases without positive virus test results). The y-axes were transformed with an exponent of 0.5 to clearly display boxplots, outliers (○), and extreme values (▼).
BALF = bronchoalveolar lavage fluid.

Further associations and correlations between unevenly distributed demographic characteristics and biomarker concentrations in serum or BALF could not be substantiated by descriptive and inductive analysis; this included the following:

- association with gender,
- association with glucocorticoids received during 14 days before bronchoscopy,
- association with T cell suppressing medications received during 3 months before bronchoscopy,
- correlation with PCT levels in serum during 4 days before/after bronchoscopy,
- association with cultural growth of pathogenic bacteria from any sample type,
- association with cultural growth of pathogenic bacteria specifically from BALF.

Furthermore, there was no evidence of an association between lower biomarker concentrations in serum or BALF and administration of mold-active antifungal therapy.

Another important factor influencing the determination of diagnostic performance values was the handling of the possible IMI group within statistical analysis. The debate regarding how cases allocated to the possible IMI group should be considered in diagnostic studies remains a subject of discussion. On the one hand, some researchers regarded this group as being overly affected by false-positive cases, erroneously labeled as IMIs, and consequently excluded them from analysis to avert an unwarranted deterioration of performance metrics. On the other hand, other researchers argued that the risk of systemic bias was too high when these cases were completely excluded; therefore, they regarded the possible IMIs/IAs as condition-positive or -negative. In most cases, possible IMIs/IAs were defined as condition-negative, for example in the systematic reviews and meta-analyses of the renowned Cochrane Collaboration (226, 231, 274). Generally, the possible IA group comprises only a low number of true-positive cases, thus causing a significant negative effect on sensitivity by increasing the count of false-negative cases when assigned to the condition-positive group (226, 231). Conversely, specificity commonly exhibits only a marginal decline when possible IAs are considered condition-negative. The complete exclusion of possible IAs generally results in better sensitivity and specificity, potentially leading to an overestimation of test performance metrics. Considering the uncertainty that cannot be excluded, the classification of possible IMI/IA cases as condition-negative is a compromise intended to have the least biasing effect. However, to provide a comprehensive reflection of this situation, the performance analyses for the other variants of classification are also enumerated in Table 40.

Table 40 *Influence on Test Performances by Possible IMI Cases*

Allocation of possible IMIs	Sensitivity [95% CI]	Specificity [95% CI]	LR+ [95% CI]	LR– [95% CI]	DOR [95% CI]
Interleukin 8 (bronchoalveolar lavage fluid) ≥ 877.7 pg/mL					
Condition negative (up vs. pp)	74% [52%, 90%]	63% [53%, 73%]	2.00 [1.36, 2.94]	0.42 [0.19, 0.90]	4.81 [1.59, 14.61]
Condition positive (u vs. ppp)	54% [39%, 68%]	64% [51%, 75%]	1.47 † [0.95, 2.25]	0.73 † [0.51, 1.06]	2.00 † [0.91, 4.40]
Excluded (u vs. pp)	74% [52%, 90%]	64% [51%, 75%]	2.02 [1.32, 3.08]	0.41 [0.19, 0.90]	4.87 [1.55, 15.27]
Interleukin 6 (bronchoalveolar lavage fluid) ≥ 55.7 pg/mL					
Condition negative (up vs. pp)	84% [64%, 96%]	49% [39%, 60%]	1.67 [1.25, 2.21]	0.32 [0.11, 0.92]	5.21 [1.42, 19.18]
Condition positive (u vs. ppp)	65% [50%, 78%]	49% [37%, 61%]	1.28 † [0.92, 1.78]	0.71 † [0.44, 1.15]	1.81 † [0.81, 4.02]
Excluded (u vs. pp)	84% [64%, 96%]	49% [37%, 61%]	1.66 [1.21, 2.26]	0.32 [0.11, 0.93]	5.17 [1.37, 19.50]
Interleukin 8 (serum) ≥ 55.6 pg/mL					
Condition negative (up vs. pp)	47% [26%, 69%]	90% [82%, 95%]	4.58 [2.10, 9.98]	0.59 [0.38, 0.91]	7.80 [2.51, 24.56]
Condition positive (u vs. ppp)	30% [18%, 45%]	92% [84%, 97%]	3.81 [1.47, 9.91]	0.76 [0.61, 0.94]	5.03 [1.64, 15.43]
Excluded (u vs. pp)	47% [26%, 69%]	92% [84%, 97%]	5.97 [2.27, 15.67]	0.57 [0.37, 0.88]	10.44 [2.90, 37.65]

Allocation of possible IMIs	Sensitivity [95% CI]	Specificity [95% CI]	LR+ [95% CI]	LR– [95% CI]	DOR [95% CI]
Aspergillus polymerase chain reaction (bronchoalveolar lavage fluid)					
Condition negative (up vs. pp) [a]	16% [4%, 36%]	98% [93%, 100%]	6.47 [1.16, 36.09]	0.86 † [0.71, 1.05]	7.50 [1.16, 48.56]
Condition positive (u vs. ppp) [b]	10% [3%, 21%]	98% [93%, 100%]	5.62 † [0.65, 48.49]	0.92 † [0.83, 1.02]	6.12 † [0.66, 56.73]
Excluded (u vs. pp) [c]	16% [4%, 36%]	98% [93%, 100%]	9.32 [1.03, 84.36]	0.86 † [0.70, 1.04]	10.88 [1.06, 111.76]
Aspergillus Lateral-Flow Device (bronchoalveolar lavage fluid)					
Condition negative (up vs. pp)	42% [22%, 64%]	97% [91%, 99%]	12.21 [3.57, 41.80]	0.60 [0.41, 0.88]	20.36 [4.69, 88.41]
Condition positive (u vs. ppp)	19% [9%, 32%]	95% [88%, 99%]	3.91 [1.10, 13.90]	0.86 † [0.73, 1.00]	4.57 [1.14, 18.37]
Excluded (u vs. pp)	42% [22%, 64%]	95% [88%, 99%]	8.84 [2.60, 30.06]	0.61 [0.41, 0.90]	14.55 [3.33, 63.54]

Note. The stated performance values apply to the diagnosis of invasive mold infections (IMI). IMIs were categorized according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC)/National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) consensus definitions. The three constellations evaluated were composed as follows: unlikely/possible IMIs (n_{up} = 87) versus probable/proven IMIs (n_{pp} = 19; up vs. pp); unlikely IMIs (n_{u} = 63) versus possible/probable/proven IMIs (n_{ppp} = 43; u vs. ppp); and unlikely IMIs (n_{u} = 63) versus probable/proven IMIs (n_{pp} = 19; u vs. pp). N = 106 (63 unlikely IMIs, 24 possible IMIs, 19 probable/proven IMIs). CI = confidence interval; DOR = diagnostic odds ratio; LR+ = positive likelihood ratio; LR– = negative likelihood ratio.

[a] N = 101: n_{up} = 82, n_{pp} = 19. [b] N = 101: n_{u} = 59, n_{ppp} = 42. [c] N = 78: n_{u} = 59, n_{pp} = 19.

† Not significant, as the 95% CI included the value 1.00.

Implementing the adaption of the mycological criteria of the 2020 EORTC-IDG/ MSGERC consensus definition (18) into our case categorization process would have resulted in the reclassification of some cases as follows:

- Six probable IMI cases would have been designated as possible IMI cases if BDG had been excluded from the mycological criteria (and cultural growth of molds from skin punch samples had not been accepted as mycological criterion).
- One probable IMI/IPA case would have been reclassified to the possible IMI/IPA group, as galactomannan thresholds had been specified at a higher level than the thresholds defined by the manufacturers that had been accepted until the 2020 revision.
- Conversely, two possible IMI/IPA cases would have been classified as probable IMI/IPA cases despite the higher serum galactomannan thresholds if a single positive galactomannan test had been sufficient instead of two consecutive positive measurements.

Finally, these changes would result in 14 probable/proven IMIs. According to ROC analysis, IL-6 and IL-8 in BALF as well as IL-8 in serum would still demonstrate discriminatory potential; however, the optimal cut-offs would be shifted upward at 136.5 pg/mL for IL-6 in BALF, at 954.2 pg/mL for IL-8 in BALF, and downward at 13.7 pg/mL for IL-8 in serum. The resulting test characteristics are listed in Table 41, which also includes the characteristics of the *Aspergillus* PCR and AspLFD that resulted from adaptation of the consensus definitions. In addition, Table 41 lists the performance data that would have been obtained if we had not applied slightly stricter requirements for the 2008 EORTC/MSG mycological criteria, namely, two subsequent positive test results when incorporating galactomannan tests with serum. As already mentioned in the methods section, this additional requirement is known to improve the specificity of the 2008 EORTC/MSG consensus definitions in its role as reference standard but without compromising sensitivity (226, 292). However, this was criticized by individual reviewers who reviewed our previous publications as a biasing deviation from the 2008 EORTC/MSG criteria. There would have been two additional probable IMI cases in our study cohort (n = 21) if only one positive galactomannan result with serum had been required for defining probable IMIs.

Table 41 *Influence of Changes in Consensus Definitions on Test Performance*

Test	Sensitivity	Specificity	LR+	LR–	DOR
2020 EORTC-IDG/MSGERC consensus definition [a]					
Serum					
Interleukin 8 ≥ 55.6 pg/mL	38%	86%	2.52	0.75 †	3.38 †
Interleukin 8 ≥ 13.7 pg/mL	79%	59%	1.90	0.37 †	5.21
Bronchoalveolar lavage fluid					
Interleukin 8 ≥ 877.7 pg/mL	71%	61%	1.83	0.47 †	3.89
Interleukin 8 ≥ 954.2 pg/mL	71%	63%	1.93	0.45 †	4.27
Interleukin 6 ≥ 55.7 pg/mL	93%	49%	1.82	0.15	12.45
Interleukin 6 ≥ 136.5 pg/mL	86%	58%	2.02	0.25	8.15
Aspergillus PCR [b]	21%	98%	9.32	0.80 †	11.59
Aspergillus Lateral-Flow Device	50%	96%	11.50	0.52	22.00
2008 EORTC/MSG consensus definition without our more stringent serum GM criterion [c]					
Serum					
Interleukin 8 ≥ 55.6 pg/mL	48%	91%	5.06	0.58	8.75
Bronchoalveolar lavage fluid					
Interleukin 8 ≥ 877.7 pg/mL	67%	62%	1.77	0.55 †	3.31
Interleukin 6 ≥ 55.7 pg/mL	81%	49%	1.60	0.39	4.15
Aspergillus PCR [d]	14%	98%	5.71	0.88 †	6.50
Aspergillus Lateral-Flow Device	38%	97%	10.79	0.64	16.82

Note. Test performance metrics if the cases included in this study had been categorized (a) according to the 2020 European Organization for Research and Treatment of Cancer/ Infectious Diseases Group (EORTC-IDG)/Mycoses Study Group Education and Research Consortium consensus definitions (MSGERC), or (b) according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC)/National Institute of Allergy and Infectious Diseases Mycoses Study Group (MSG) consensus definitions without our adaption to incorporation of serum galactomannan (GM) results (i.e., necessitating two consecutive positive test results). Unlikely and possible invasive mold infections (IMI) were defined as disease-negative, probable/proven IMIs were

defined as disease-positive. *N* = 106. DOR = diagnostic odds ratio; LR– = negative likelihood ratio; LR+ = positive likelihood ratio; PCR = polymerase chain reaction.
[a] Defining 92 unlikely/possible IMIs and 14 probable/proven IMIs. [b] *N* = 101 (87 unlikely/possible IMIs, 14 probable/proven IMIs). [c] Defining 85 unlikely/possible IMIs and 21 probable/proven IMIs. [d] *N* = 101 (80 unlikely/possible IMIs, 21 probable/proven IMIs).
[†] The 95% confidence interval included the value 1.00.

However, this would not have changed the performance results relevantly: All point estimators would have been within the 95% CI of our main analysis, although IL-6 and IL-8 in the BALF were estimated slightly more optimistically in the main analysis. Even when using the 2020 EORTC-IDG/MSGERC consensus definitions, all point estimators were within the 95% CIs of our main analysis, except for IL-8 in serum after adjustment of its optimal cut-off. When using the 2020 EORTC-IDG/MSGERC definitions as the reference standard, IL-8 tended generally to exhibit poorer performance values, but IL-6, the *Aspergillus* PCR, and the AspLFD tended to demonstrate better performance values. Better diagnostic performance of the *Aspergillus* PCR and the AspLFD were not surprising, as the changes in the 2020 EORTC-IDG/MSGERC definitions led to a stronger weighting of IPA within the probable/proven IMI group, since BDG was no longer included and non-galactomannan containing/non-*Aspergillus* molds could only be included via culture, microscopy, biopsy, or autopsy anymore. To diagnose IMIs, however, one pattern remained the same regardless of which variant of the reference standard was applied to our data: IL-6 in BALF offered the best sensitivity and negative LR, thus being the best option to rule-out an IMI; the *Aspergillus* PCR with BALF offered the best specificity but at the same time did not reveal a significant negative LR in any variant; and the AspLFD with BALF offered the best positive LR and DOR, with the second-best specificity, thus being the best option to rule-in IPAs and—depending on the local epidemiology—also IMIs, respectively.

Discussion

The primary purpose of this prospective cohort study was to evaluate the diagnostic potential of a selection of immunologic biomarkers. Our study cohort represented a high-risk population that consisted exclusively of patients with underlying hematological malignancies, including patients with myeloid malignancies, patients that underwent allogeneic HCTs, and patients with subsequent GVHDs. The diagnostic situation under mold-active antifungal medication is particularly well represented, since more than 80% of the study participants received such medications at the time of sample collection, and almost all of them for at least 2 days. Regarding immunologic biomarkers, we could ultimately demonstrate only for IL-8 in serum as well as for IL-6 and IL-8 in BALF a potential to distinguish between unlikely/ possible IMIs and probable/proven IMIs. Regarding *Aspergillus*-specific tests, the AspLFD performed with BALF demonstrated superior diagnostic performance in comparison to our *Aspergillus* PCR assay.

Concentrations of Cytokines and Chemokines

In two previous publications, our research group has already evaluated the present set of biomarkers regarding their potential to diagnose IPAs (81, 82). Additional studies that evaluated concentrations of immunologic biomarkers for IMIs in clinical settings were conducted by Roilides et al. (124) (i.e., IL-10, IL-12, TNF-α in serum), Ellis et al. (91) (i.e., CCL5 in serum), Chai et al. (107) (i.e., IL-17 in serum and BALF), Radowsky et al. (92) (e.g., CCL5, sIL-2R, and IL-15 in serum), Ceesay et al. (164) (i.e., 30 biomarkers in serum, including our selection except IL-22), Goncalves et al. (83) (i.e., 32 biomarkers in BALF, including our selection), He et al. (323, 324) (i.e., IL-17 in plasma and BALF), and Aerts et al. (325) (i.e., 92 biomarkers in serum, including our selection).

Comparison With Our Previous Publications

In 2017 and 2018, our research group published results demonstrating that IL-6 and IL-8 in serum and in BALF could distinguish between IPA and non-IA pneumonia (81, 82). The major advantage of these publications was that the control groups also consisted of patients with suspected pneumonia, and not just healthy controls as with most previously published studies.

The data set analyzed in this thesis is almost identical to that of the 2018 study (82). However, the present analysis was aimed at answering the question if the immunological

biomarkers are suitable for diagnosing IMIs in general. Therefore, BDG was included as a mycological criterion to classify IMI probability. This affected some cases of the possible IPA group, assigning them to the probable/proven IMI group in the current analysis. Additionally, one former possible IPA became a proven IMI (probable invasive pulmonary cladosporiosis) due to autopsy results additionally received subsequently. Although these reclassifications make the probable/proven IMI group more inhomogeneous, the analysis of this thesis also confirmed the observations that IL-8 concentrations in serum and BALF have a potential for distinguishing IMIs from non-mold pneumonias in high-risk settings for IFIs. However, the discriminatory potential of IL-8 appeared to be less strong compared to its potential in IPAs, and the potential of IL-6 in serum could not be confirmed finally (82). Regarding IL-6, the differences compared to our former publication can be explained by the different case classifications. When BDG had been considered for the definition of probable IMI, more cases with lower serum IL-6 levels were classified in this category, resulting in changes in AUCs of ROC analysis: AUC = .751, p = .008, for unlikely versus probable IPAs; compared to AUC = .616, p = .126, for unlikely versus probable/proven IMIs (our former publication excluded possible IPAs, therefore possible IMIs were also excluded for this comparison to reach comparability) (82). However, the discriminatory potential of IL-6 in BALF remained similar despite inclusion of BDG as a mycological criterion: AUC = .680, p = .059, for unlikely versus probable IPAs; compared to AUC = .665, p = .012, for unlikely versus probable/proven IMIs. Differences in ROC analysis can be explained by the more inhomogeneous probable/proven IMI group compared to groups representing only one mold genus; different fungal genera can stimulate the release of cytokines and chemokines to different extents, as demonstrated for the release of TNF-α and IL-6 from monocytes in vitro (78, 121). Another possibility is that false-positive BDG values led to non-IMI cases being classified as probable IMIs. The 2020 EORTC-IDG/MSGERC definitions considered the problems with the specificity of BDG and excluded BDG as a mycologic criterion for defining IMIs (127). However, recruitment of our cohort was finished prior to 2020, therefore cases were classified in agreement with the 2008 EORTC/MSG criteria that were valid at that time; furthermore, post-hoc changing of the criteria could introduce bias (e.g., misclassification due to exclusion of BDG without exhausting the expansion of criteria by including *Aspergillus* PCR screening) (125).

Comparison with Publications of Other Research Groups

Goncalves et al. (83) demonstrated that IL-8, IL-6, and IL-17A in serum and BALF, and TNF-α in BALF had diagnostic potential. We could not confirm the observations on TNF-α, but we also found differences of IL-17A concentrations in the BALF between our unlikely and probable/proven IMI cases. Medians and IQR of IL-17A in BALF that were observed in our study were similar to those of Goncalves et al., but the range was broader within the data of Goncalves et al. (*Mdn* = 1.9 pg/mL, IQR = 5.8, range = 34.7 regarding probable/ proven IPAs; and *Mdn* = 0.0 pg/mL, IQR = 5.0, range = 12.4 regarding probable/proven IMIs). One reason may be that the IPA group of Goncalves et al. also included patients without underlying hematological diseases. Presuming that these patients had been less immunocompromised, they could have been able to build up higher cytokine levels. In addition, Goncalves et al. had excluded cases with antifungal therapies; this could have affected the composition of the study population (e.g., affecting severity/invasiveness or dynamics of IPAs, or the proportion of mixed IMIs within the study population) (83). Similarly, He, Li, et al. (324) and He, Cao, et al. (323) also published studies demonstrating higher IL-17 levels in BALF of patients with various pulmonary diseases and IPAs, but without neutropenia. In contrast, Chai et al. (107) did not find higher IL-17 levels in BALF of patients with hematological malignancies and IPA with their case-control study. However, they attributed the negative findings to metabolites of *Aspergillus* (i.e., kynurenine), but not to the immunocompromised state of the hosts. It is known that kynurenine can inhibit the Th17 response. Chai et al. even found lower IL-17 levels in serum of cases with IPA in comparison to their control cases. However, Chai et al. based their association between kynurenine and low IL-17 levels on data of accompanied in vitro experiments but did not perform parallel evaluations in vivo (107).

Aerts et al. (325) also reported divergent results for concentrations of immunologic biomarker concentrations in IMIs, using a proximity extension assay. In their retrospective study design, cases with IA and underlying hematological malignancies were paired with control cases without infections. Cases with IA exhibited elevated serum levels of IL-8, IL-6, IL-17A, TNF-α, and IL-10. However, after pairing IAs with control cases that had non-fungal infections the differences were no longer significant. These findings underscore the importance of evaluating the diagnostic potential of immunological biomarkers, which are rather unspecific, in the presence of various other infections to achieve realistic settings and

real-life diagnostic problems. Moreover, Aerts et al. also conducted a prospective study arm in which cases were matched with control cases without infections; interestingly, the cytokines were also not significantly elevated in IAs in this situation, contrarily to their retrospective study arm. However, the number of samples in this prospective study arm (i.e., 20 cases in each group) was probably too low for the applied regression analysis with multiple comparisons; the study design possibly had a high β error probability (325). Unfortunately, a descriptive analysis of biomarker concentrations was not published, therefore a direct comparison with our biomarker concentrations was not possible. In summary, however, the inductive statistical analysis results of Aerts et al. did not support our findings.

Roilides et al. (124) demonstrated increasing or persistently high IL-10 levels in serum of patients with IA. In our previous publications (81, 82), we also detected higher IL-10 levels in serum of probable IPA cases compared to unlikely IPA cases with other infections; nevertheless, there was no discriminative potential in ROC analysis both times. In this thesis focusing on IMIs in general, however, there were no notable differences of IL-10 concentrations in serum. This was also most likely caused by different case categorizations, as the possible IPAs that were assigned to the probable IMI group had lower serum IL-10 levels on average.

Ceesay et al. (164) and Radowsky et al. (92) concluded that higher sIL-2R values could pose risk factors for subsequent IFIs and IMIs, respectively. During active IMIs, nevertheless, we could not demonstrate diagnostic potential for sIL-2R. Similarly to sIL-2R, Radowsky et al. described a prognostic potential for IL-15 and a prognostic and diagnostic potential for CCL5, but again we could not reinforce this with our data (92). However, the study of Ceesay et al. was focused on discovering new immunological risk factor prior to the onset of IMIs, and the study of Radowsky et al. had a retrospective case-control design with a study population that consisted of combat-wounded, non-immunocompromised individuals (92). Thus, both studies are only limitedly comparable to our study. Nevertheless, it is noteworthy that the serum levels of CCL5 found by Radowsky et al. were much higher than in our study, M = 10,492.8 pg/mL and M = 5,333.3 pg/mL in combat-wounded persons with or without IMI, respectively; compared to M = 85.1 pg/mL and M = 150.9 pg/mL in our patients with hematological malignancies and unlikely or probable/proven IMIs, respectively. Moreover, the values measured by Radowsky et al. tended to be within the normal range when compared to studies that reported CCL5 concentrations from control groups consisting

of healthy volunteers (92, 326-328). In another study on CCL5, Ellis et al. (91) described falling CCL5 concentrations in serum of 14 patients with hematological malignancies and IFIs (including 10 probable/proven IAs) after undergoing chemotherapy. In cases with IA, CCL5 concentrations were between 16 pg/mL and 7,634 pg/mL at the nadir and exhibited a correlation with platelet count. Summarizing these observations regarding CCL5 and our results, CCL5 levels in patients with hematological malignancies were strikingly low during an IMI, but, on the one hand, we could not demonstrate any difference between IMIs and, on the other hand, pneumonias caused by other pathogens in our study. Decreased CCL5 levels may possibly be a side effect of cytopenia due to a loss of CCL5-producing cells, followed by an impaired chemotaxis of immune cells, and therefore possibly posing an additional risk factor for infections in general. However, no correlation between CCL5 concentrations and leukopenia could be demonstrated with our data.

Diagnostic Performances

Of the tests to be evaluated, IL-6 and IL-8 in BALF were most effective in reducing the post-test probability of an IMI, while IL-8 in serum as well as *Aspergillus* PCR and AspLFD in BALF were most effective in increasing the post-test probability.

The optimal cut-off of IL-8 in BALF was 877.7 pg/mL, with a negative LR of 0.42, respectively (see Table 23). However, many false-positive results are to be expected, and its usefulness for reducing the post-test probability is questionable. Nevertheless, the wide 95% CI of the negative LR also covered a range down to 0.19, therefore there is a possibility that further studies could demonstrate a useful potential for reducing post-test probabilities. Contrary, the 95% CI of the positive LR indicated that IL-8 in BALF with this cut-off will likely not exhibit values that would be effective for rising post-test probabilities in further studies. The same constellation applied to IL-6 in BALF with a cut-off of 55.7 pg/mL; in fact, IL-6 in BALF exhibited a useful negative LR (0.32) and a slightly better DOR, thus promised to be the preferable choice in comparison with IL-8 when using BALF. In contrast, IL-8 in serum demonstrated a positive LR of 4.58 with a cut-off of 55.6 pg/mL, thus, a positive result can slightly increase the post-test probability (see Table 27). However, the 95% CI was broad again, and diagnostic usefulness needs to be confirmed in further studies (for both IL-6 and IL-8). Conversely, because of many false-negative results with this cut-off, a negative result by IL-8 measurements in serum was hardly meaningful, and the 95% CI indicated that this will not change substantially in further studies.

The *Aspergillus* PCR exhibited very poor performance with blood in our study and did not yield any true-positive results. This is strikingly low, but there are other studies with similar results. For example, the study with the lowest sensitivity (22%) in the review by Cruciani et al. (274) was the study from Aslan et al. (329). However, Aslan et al. even implemented a screening strategy and took samples twice weekly, whereas we evaluated a diagnostic test strategy with only a single PCR measurement per patient. This is an important difference as the amount of *Aspergillus* DNA in the blood circulation during infection is not always the same but depends on the total fungal load, current invasiveness, and the lysis of hyphae (278). Thus, the sensitivity of the PCR depends on the point in time during the infection when blood samples are taken: The more frequent samples are taken the more likely it is that this time point will be hit. However, our DNA extraction methodology was also not optimal, which could also explain some of the low sensitivity. In fact, DNA extraction is usually the decisive point for the performance of an *Aspergillus* PCR with blood samples (277). Fungal cell material within the bloodstream presumably consists of hyphal fragments (e.g., freely circulating or within phagocytes) or cell free fungal DNA (36, 38, 39). Lysis of hyphae, which results in cell free DNA, is caused by lack of nutrients, a functioning immune defense, or under antifungal therapy, among other factors (36). As 80% of our cases received antifungal therapy, it can be assumed that circulating fungal cell material comprised a high proportion of cell free DNA (278, 280). We utilized whole blood specimens, but the necessary pretreatment for this sample type is known to noticeably reduce the sensitivity of the PCR to detect cell free DNA; therefore, serum samples would have been the better choice to detect free DNA (278, 280). At the same time, our DNA extraction protocol did not include bead beating. However, mechanical destruction of hyphal cell walls via bead beating is superior to the chemical-enzymatic hyphal cell wall lysis employed with our protocol, and bead beating is also listed in the meanwhile established standardization recommendations of the Fungal PCR Initiative (FPCRI) as a relevant step for whole blood samples to ensure good sensitivity (280, 296, 297, 330). In addition, different primers utilized could also be responsible for some difference. In contrast to sensitivity, however, we observed an outstanding specificity of 100%, whereas false-positive results with blood or serum samples due to contamination would not have been extraordinary (271, 277).

The sensitivity of the *Aspergillus* PCR with BALF was slightly better than that with serum, but still far too low to be meaningful (see Table 35). Therefore, a negative PCR result

from the BALF was not informative. A possible reason for our low sensitivity with BALF is that mold-active antifungals will decrease the sensitivity of PCR when they are administered for 2 days or longer, or more than one antifungal is administered (234, 281). Whereas Cruciani et al. (274) were not able to prove this for serum by a subgroup analysis implemented in their meta-analysis, Avni et al. (275) demonstrated a lower summary sensitivity with BALF in cases receiving antifungals (i.e., 58% versus 90%, respectively; possible IPAs excluded). In addition, difficulties in standardizing BALF sampling could pose another reason for low sensitivities (234). The concentrations of antigens and DNA within BALF are prone to dilution effects as the lavage fluid cannot be fully recovered anytime, and sometimes more lavage fluid volume is applied in everyday clinical practice to have sufficient BALF for all necessary diagnostics procedures. Furthermore, different volumes of the airways lavaged introduce variability to concentrations in BALF samples. Additionally, loss of cell free DNA of the BALF was also to be expected with our DNA extraction method used, and this was again coupled with the absence of bead beating, which is recommended for an efficient release of fungal DNA from retrieved *Aspergillus* hyphae in BALF (278, 280, 330). The specificity of the *Aspergillus* PCR with BALF determined in this study, on the other hand, corresponds to the range to be expected from the literature, and a positive result was able to moderately increase the post-test probability of an IMI (275). Nevertheless, false-positive results still occurred and are known to be caused by contamination of samples or by colonization of the respiratory tract with *Aspergillus* (331). However, due to the lack of a reliable gold standard, there is also the possibility that a true-positive result is assessed as false-positive.

We also did not obtain any true-positive result with the AspLFD with serum. False-negative results can occur due to inhibition of the assay by serum proteins if serum is not pretreated appropriately (284). However, we followed the manufacturer's instructions. Another explanation would again be the antifungal therapy, which is assumed to noticeably reduce the sensitivity of the AspLFDs, as the targeted antigen is only released during active fungal growth (224, 287). Another study on the performance of the AspLFD, in which every patient received antifungal therapy, was conducted by Held et al. (224) who reported a sensitivity of 40%. However, this estimation again reflected serial measurements. Furthermore, White et al. (286) demonstrated that the AspLFD with serum was less sensitive than the PCR with whole blood. In conclusion, it is not surprising not to achieve any

true-positive result with the AspLFD with serum in a setting with single measurements, high prevalence of mold-active antifungal prophylaxis or therapy, and no true-positive results with the *Aspergillus* PCR. Contrary, our specificity of 98% was very high compared to the values determined by Pan et al. (284). Regarding false-positive results, the AspLFD cross-reacted to *Paecilomyces variotii* and to some *Penicillium spp.* (but not including *Talaromyces marneffei*, formerly named *Penicillium marneffei*) (332). However, infections by *Paecilomyces* or *Penicillium* are rare even within high-risk situations (19, 332). When misclassification occurs due to the imperfection of the EORTC/MSG definitions, officially false-positive AspLFD results may in fact be true-positives; our two positive AspLFDs with serum belonged to the unlikely and possible IMI/IPA groups, thus were interpreted as false-positive results. However, we cannot offer evidence to interpret them as actually true-positive results. Both cases received screening for IMIs with galactomannan and BDG in serum. Screening tests remained negative and were not close to the cut-off limits around the time the AspLFD was performed. Galactomannan, *Aspergillus* PCR, and AspLFD with BALF also remained negative. CRP levels were significantly elevated in both cases. Other microbiological tests only revealed viruses as possibly relevant infectious pathogens (i.e., EBV, CMV, influenza A). In CT scans, the possible IMI/IPA case presented multiple small consolidations subpleural in both lungs. The patient had not received antifungal prophylaxis and received voriconazole therapy for only 1 day before serum sample collection for the AspLFD. The unlikely IMI/IPA case had a non-specific pulmonary infiltrate but also evidence for sinusitis in the CT scan. However, it was not possible to either confirm or exclude fungal etiology by CT, and microbiological work-up with samples from sinuses was not performed in clinical routine. Nevertheless, if the case is assigned to the possible IMI/IPA category, it will still not change the classification of the AspLFD result as a false-positive one. Regarding antifungals, the patient received long-term prophylaxis with posaconazole.

With BALF, the AspLFD had a sensitivity of 73% for the diagnosis of IPA; this was slightly lower than the sensitivity (86%) calculated in the meta-analysis by Pan et al. (284). However, our sensitivity of this study was consistent with (a) the 95% CI [59%, 100%] calculated by Pan et al. in a subgroup analysis of studies that only comprised patients with hematological malignancies, and (b) our own estimation of 67% sensitivity calculated and published in our former review about lateral-flow assays for IA (287). In fact, the sensitivity determined with our cohort was not reduced but rather relatively high, as a loss in sensitivity

of 20%–30% can be expected in presence of a high proportion of antifungal therapy (284, 287). Due to its specificity for *Aspergillus*, sensitivity decreases to 42% when the diagnosis in question is an IMI in general (see Table 35 and Table 42) (332). Specificity was high (i.e., 95%–97%, depending on inclusion of BDG for case categorization), and covered by the 95% CI [79%, 100%] calculated by the subgroup analysis of Pan et al. (82, 284, 287). The specificity is also the strength of this assay. A positive AspLFD test performed with BALF substantially increased the post-test probability for an IMI/IPA. In contrast to utilizing the AspLFD in diagnostics for IPA, however, lowering post-test probability of an IMI in general was not sufficiently possible with the AspLFD; this ability depends particularly on the proportion of non-*Aspergillus* IMIs within the population under investigation and the local epidemiologic situation, respectively: The higher the proportion of IPAs, the higher the sensitivity of the AspLFD to detect IMIs, and the lower (i.e., better) its negative LR.

Comparison of Diagnostic Tests

The sole proven IMI case in our cohort was only identified through autopsy, which included a histopathological evaluation and subsequent PCR for the identification of microscopically visible hyphae. This underscores that biopsies and their work-up via microscopy, culture, and PCR remain important diagnostic modalities for IMIs. Furthermore, it also highlights the value of autopsies for diagnostic studies of IMIs and for monitoring the local incidence of non-*Aspergillus* IMIs. Notably, levels of both IL-8 and IL-6 in BALF of this case were elevated above the suggested cut-offs and at the same time, they were the only positive test results at time of bronchoscopy; however, they did not reach the highest levels observed within the probable/proven IMI group.

The sensitivity of conventional cultures was found to be very limited (i.e., 5% for BALF samples). In both probable IMIs with molds growing in respiratory samples, *Scedosporium apiospermum* was identified. IL-8 and IL-6 in BALF were clearly positive both times. Among the established biomarkers, only BDG indicated an IFI in these cases. This is interesting because the value of BDG for diagnosing *Scedosporium spp.* is not well established, and this genus is known from in vitro experiments to release rather little amounts of BDG (249, 250). However, the study data gave no explanation for the presence of another microbial source of BDG. Nevertheless, false-positive BDG results or the presence of an undetected concurrent fungal infection cannot be ruled out.

Table 42 *Overview Over Test Performances*

Test and cut-off	Target condition	Sample material	Sens	Spec	YI	LR+	LR–	DOR
BDG ≥ 80 pg/mL [a]	IFI	Serum	80%	63%	.43	2.16	0.32	6.81
BDG ≥ 80 pg/mL [b, c]	IPA	Serum	46%	76%	.22	1.88	0.72	2.61
GM ≥ 0.5 ODI [d]	IA	Serum	78%	85%	.63	5.20	0.26	20.09
GM ≥ 1.0 ODI [d]	IA	Serum	71%	90%	.61	7.10	0.32	22.03
GM ≥ 0.5 ODI [e]	IPA	BALF	88%	81%	.69	4.63	0.15	31.26
GM ≥ 1.0 ODI [e]	IPA	BALF	78%	93%	.71	11.14	0.24	47.10
Aspergillus PCR [f]	IA	Whole blood, serum	79%	80%	.59	3.88	0.26	15.05
Aspergillus PCR [g]	IPA	BALF	90%	96%	.86	25.06	0.10	216.00
Aspergillus PCR [b, h]	IPA	BALF	27%	98%	.25	12.27	0.74	16.50
Aspergillus PCR [b, i]	IMI	BALF	16%	98%	.14	6.47	0.86	7.50
AspLFD [j]	IA	Serum	68%	81%	.49	3.58	0.40	8.95
AspLFD [j]	IPA	BALF	86%	84%	.70	5.38	0.17	31.65
AspLFD [b, c]	IPA	BALF	73%	97%	.70	23.03	0.28	81.78
AspLFD [b, k]	IMI	BALF	42%	97%	.39	12.21	0.61	20.36
IL-8 ≥ 55.6 pg/mL [b, c]	IPA	Serum	55%	87%	.42	4.32	0.52	8.30
IL-8 ≥ 55.6 pg/mL [b, k]	IMI	Serum	47%	90%	.37	4.58	0.59	7.80
IL-8 ≥ 877.7 pg/mL [b, c]	IPA	BALF	73%	60%	.33	1.82	0.46	4.00
IL-8 ≥ 877.7 pg/mL [b, k]	IMI	BALF	74%	63%	.37	2.00	0.42	4.81
IL-6 ≥ 55.7 pg/mL [b, c]	IPA	BALF	91%	47%	.38	1.73	0.19	9.00
IL-6 ≥ 55.7 pg/mL [b, k]	IMI	BALF	84%	49%	.34	1.67	0.32	5.21

Note. The performance metrics are based on calculations that regarded unlikely/possible invasive fungal infections (IFI) as condition-negative and probable/proven IFIs as condition-positive, unless otherwise indicated. The sensitivities and specificities from meta-analyses that refer to whole blood and serum samples were predominantly based on studies that implemented serial measurements; the other performance metrics were predominantly based on studies that implemented single measurements. The Youden's indices (YI), positive likelihood ratios (LR+), negative likelihood ratios (LR–), and diagnostic odds

ratios (DOR) that are reported for meta-analyses were calculated based on the sensitivities and specificities published by the meta-analyses. *Aspergillus* polymerase chain reaction (PCR) and *Aspergillus* Lateral-Flow Device (AspLFD) with whole blood or serum did not exhibit true-positive results in our study. BALF = bronchoalveolar lavage fluid; BDG = 1,3-β-D-glucan; GM = galactomannan; IA = invasive aspergillosis; IL = interleukin; IPA = invasive pulmonary aspergillosis; ODI = optical density index; Sens = sensitivity; Spec = specificity.

[a] Based on the sensitivity and specificity published by White et al. (253) that were calculated from low-bias studies only. [b] Metrics were calculated with our data. [c] $N = 106$ (95 unlikely/possible and 11 probable IPAs). [d] Based on the sensitivity and specificity published by Leeflang et al. (226). [e] Based on the sensitivity and specificity published by de Heer et al. (231). [f] Based on the sensitivity and specificity published by Cruciani et al. (274). [g] Based on the sensitivity and specificity published by Avni et al. (275); possible IPAs were excluded. [h] $N = 101$ (59 unlikely, 31 possible, and 11 probable IPAs). Possible IPAs were included; excluding possible IPAs only changed the specificity in the decimal places, but with a resulting higher LR+ = 16.09. [i] $N = 101$ (59 unlikely IMIs, 23 possible IMIs, 19 probable IMIs). Possible IMIs were included; excluding possible IMIs only changed the specificity in the decimal places, but with a resulting higher LR+ = 9.32. [j] Based on the sensitivity and specificity published by Pan et al. (284). [k] $N = 106$ (87 unlikely/possible and 19 probable/proven IMIs).

Regarding cultures derived from tissue biopsies, *Aspergillus fumigatus* was isolated from a skin punch sample in one probable IPA/IMI case. However, subsequent autopsy failed to verify the presence of an IA/IMI. Furthermore, galactomannan was negative in both serum and BALF. However, BDG was positive and, in fact, BDG had a higher sensitivity (i.e., 46%) to detect IA in our cohort than galactomannan in serum (i.e., 27% sensitivity, whereby this may be an overestimation due to a possible incorporation bias and serial measurements); furthermore, the levels of IL-8 in serum and BALF as well as IL-6 in BALF were above our suggested cut-off levels. In summary, IL-6 and IL-8 in BALF were able to indicate each IMI in our cohort that exhibited evidence by culture or autopsy; thus, they were a fast and complementary way—besides BDG—for identifying 21% (4/19) of our probable/proven IMIs.

Regarding the further IPA cases that had negative results of galactomannan and BDG in serum but had positive galactomannan results in BALF, IL-8 in serum was able to detect 50% (2/4) of these. Thus, IL-8 in serum was the fastest way for identifying IMIs in further 10% (2/19) of our probable/proven IMIs.

Table 42 presents a comprehensive overview of test characteristics obtained from meta-analyses and those calculated from our data (231, 253, 274, 275, 284). However, it is important to note that meta-analyses regarding BDG, galactomannan, *Aspergillus* PCR, and AspLFD performed with whole blood or serum were predominantly based on studies with serial measurements; serial measurements result in better test performance metrics in comparison to single measurements. Nevertheless, when comparing tests with serum with each other, galactomannan will be the test with the best overall performance and highest DOR for diagnosing IA, at least when implemented as a screening test. IL-8 in serum was found to be inferior to galactomannan due to its limited sensitivity. However, the performance of IL-8 to diagnose IAs with serum was (a) superior to BDG, (b) comparable to the AspLFD in both confirming and refuting IAs (when considering data from Pan et al. (284)), and (c) comparable to the *Aspergillus* PCR in confirming IAs (when considering data from Cruciani et al. (274)). Because BDG and galactomannan were utilized to define IMIs, it was precluded to directly compare their performances in diagnosing IMIs with that of IL-8; such a comparison would comprise an incorporation bias, favoring BDG and galactomannan.

With BALF, the best performing test to diagnose IPAs in our study was the AspLFD, with a sensitivity and specificity that were comparable to galactomannan when implementing a cut-off of 1.0 ODI. Considering data from Avni et al. (275), however, the best performing tests to diagnose IPAs with BALF are standardized *Aspergillus* PCR assays. Nevertheless, IL-6 and IL-8 in BALF may be complementing methods in diagnostics for non-*Aspergillus* IMIs, whereas IL-6 exhibited the better performance metrics to decrease and IL-8 the better metrics to increase post-test probability.

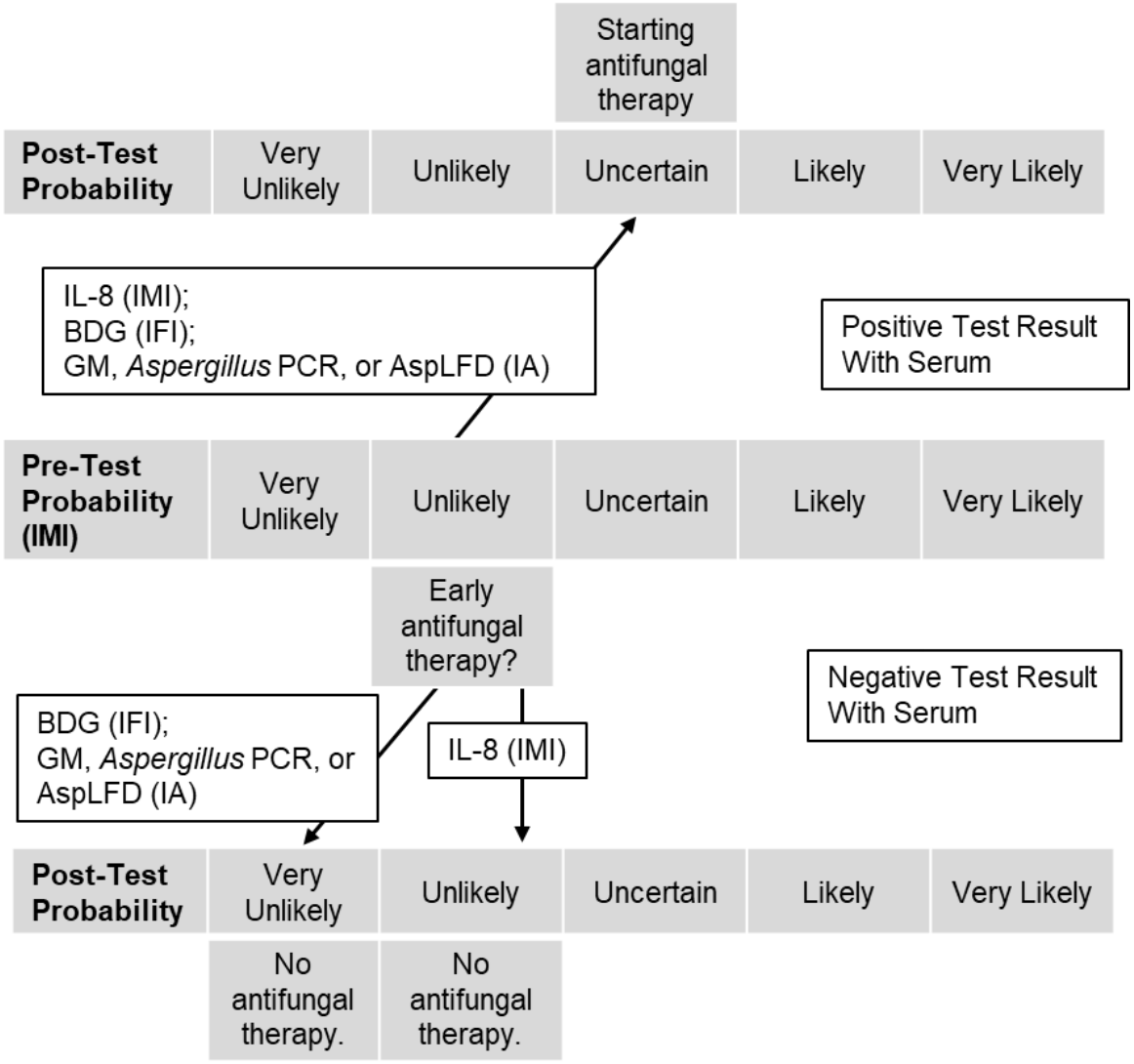
Possible Impact of Diagnostics on Clinical Practice

If a patient at high risk for IMI develops a febrile infection, the pre-test probability of an IMI is still quite low, as most infections are caused by bacteria in this situation (333). If we consider the possible effects of a diagnostic test on the post-test probability based on the practical approach of Power et al. (320) and Medow and Lucey (319) (i.e., five pre- and post-test probability levels: very unlikely, unlikely, uncertain, likely, and very likely), IL-8 in

serum can be helpful making a decision about early mold-active empirical antifungal therapy, even with a low pre-test probability (see Figure 69 and Figure 70). However, low serum IL-8 concentrations cannot lower post-test probabilities regarding IMIs. Nevertheless, a combination of IL-8, BDG, and galactomannan with serum could be utilized to early indicate a need for empiric therapy and to narrow down fungal differential diagnoses. In our study, the *Aspergillus* PCR and AspLFD with serum were not useful diagnostic tests; regarding data from meta-analyses, the AspLFD and *Aspergillus* PCR performed much better, but also had poorer overall performance compared to galactomannan with serum (see Table 42). However, particularly the results of the AspLFD will quickly be available and, therefore, the AspLFD will be a useful complementary test when galactomannan results are not available on the same day of sampling.

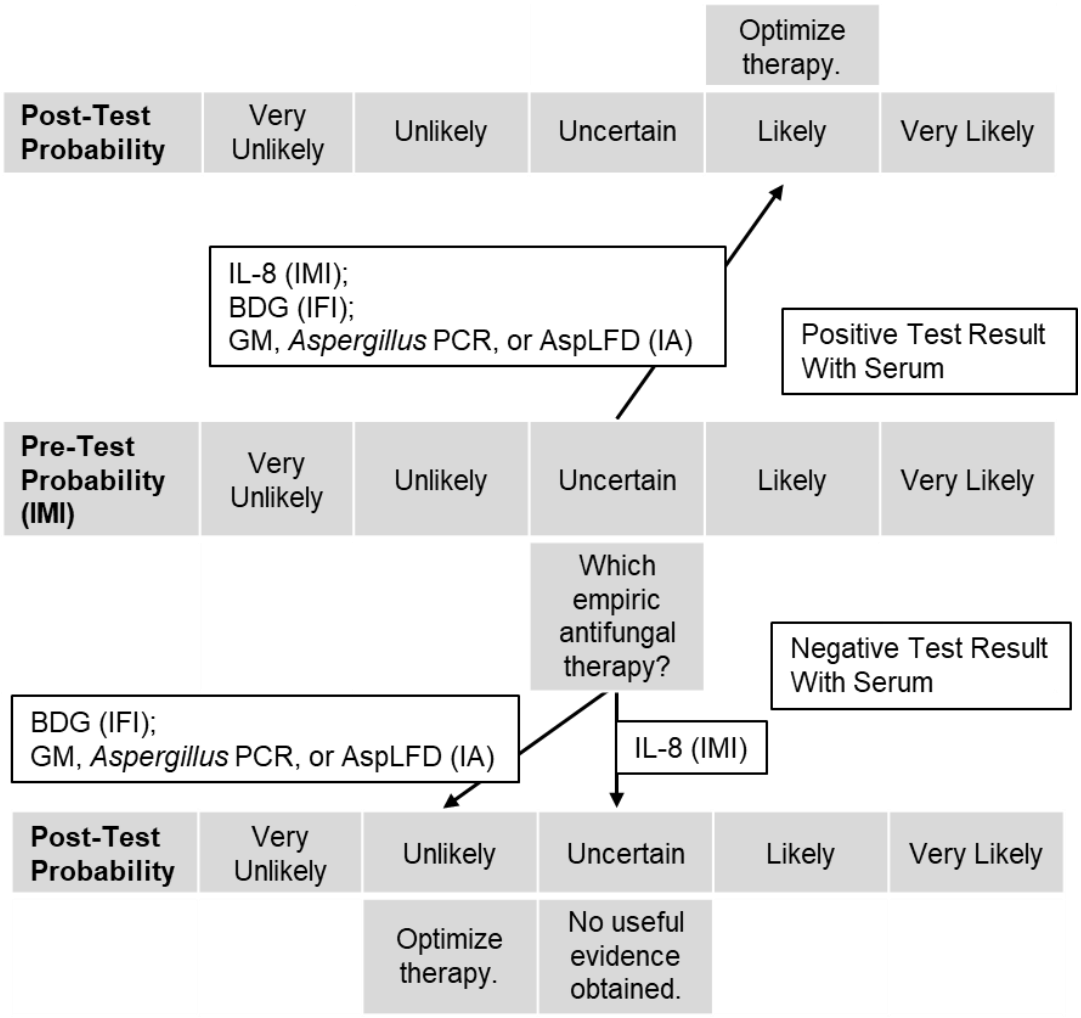
If a CT reveals changes that are characteristic of an IMI and serum diagnostics remain inconclusive, an empirical therapy will still be initiated at the latest at this point. In these cases, the primary diagnostic requirements are (a) to identify the causative mold species, including possible resistances, or (b) to identify a false-positive interpretation of the radiological findings, including the true entity that has caused the alterations in CT. For these diagnostic purposes, a bronchoscopy is still essential, including the classic work-up implementing histopathology, cytology, cultures, antigen identification, and molecular methods. Unfortunately, IL-6 and IL-8 in BALF were not suitable for such a precise differentiation of etiology and also failed to make an IMI sufficiently unlikely; thus, it is not possible to change or downscale antifungal therapies without risk by interpretation of IL-6 or IL-8 concentrations in BALF in this situation (see Figure 71). Similarly, a negative galactomannan test or AspLFD with BALF (viewed in isolation) cannot rule-out IPA in the presence of a high pre-test probability for an IPA. The *Aspergillus* PCR is said to be the most accurate test for excluding IPAs. Therefore, the *Aspergillus* PCR is currently the most helpful test with BALF in this diagnostic situation; however, we were unable to confirm this in our study.

Figure 69 *Impact of Tests With Serum When the Probability of an IMI is Low*



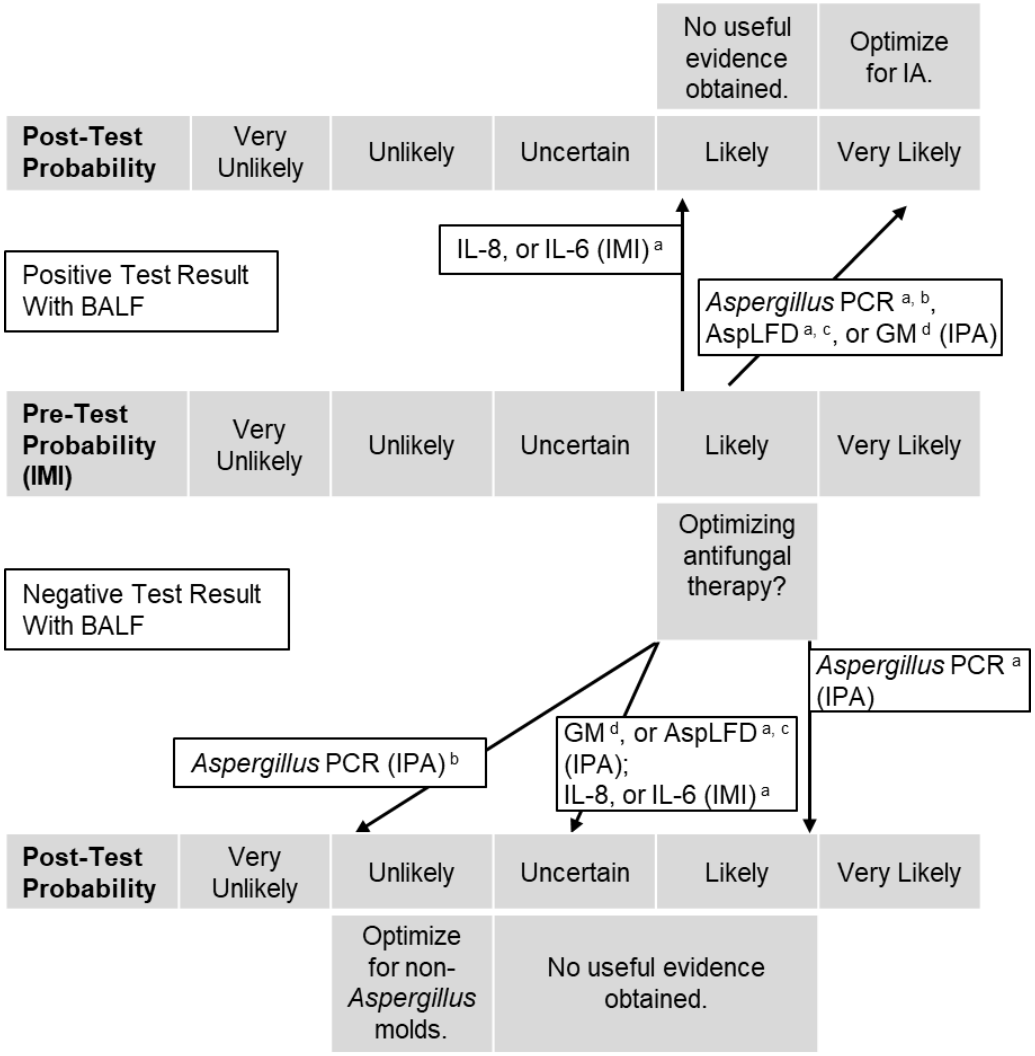
Note. This chart illustrates the influence of a single test result with serum on a low pre-test probability for an invasive mold infection (IMI) and on the possible impact on initiating a mold-active antifungal therapy. The abbreviations in brackets indicate the differential diagnosis for which the probability has been changed by the test. Categories for probabilities were adopted from Medow & Lucey (319) (i.e., unlikely pre-test probability = 20%; post-test probabilities: very unlikely = 0%–9%, unlikely = 10%–33%, uncertain = 34%–66%). The underlying likelihood ratios were retrieved from the following sources: For galactomannan (GM; 0.5 optical density index) from Leeflang et al. (226), for 1,3-β-D-glucan (BDG) from White et al. (253), for *Aspergillus* polymerase chain reaction (PCR) from Cruciani et al. (274), for *Aspergillus* Lateral-Flow Device (AspLFD) from Pan et al. (284), and for interleukin (IL) 8 from this thesis. IA = invasive aspergillosis; IFI = invasive fungal infection.

Figure 70 *Impact of Tests With Serum When the Probability of an IMI is Moderate*



Note. This chart illustrates the influence of a single test result with serum on a moderate pre-test probability for an invasive mold infection (IMI) and on the decision which mold-active antifungal agent will be started or changed (because of the high mortality of IMIs, an uncertain pre-test probability necessitates already a mold-active therapy in most cases). The abbreviations in brackets indicate the differential diagnosis for which the probability has been changed by the test. Categories for probabilities were adopted from Medow & Lucey (319) (i.e., unlikely pre-test probability = 50%; post-test probabilities: unlikely = 10%–33%, uncertain = 34%–66%, likely = 67%–90%). The underlying likelihood ratios were retrieved from the following sources: For galactomannan (GM; 0.5 optical density index) from Leeflang et al. (226), for 1,3-β-D-glucan (BDG) from White et al. (253), for *Aspergillus* polymerase chain reaction (PCR) from Cruciani et al. (274), for *Aspergillus* Lateral-Flow Device (AspLFD) from Pan et al. (284), and for interleukin (IL) 8 from this thesis. IA = invasive aspergillosis; IFI = invasive fungal infection.

Figure 71 *Impact of Tests With BALF When the Probability of an IMI is High*

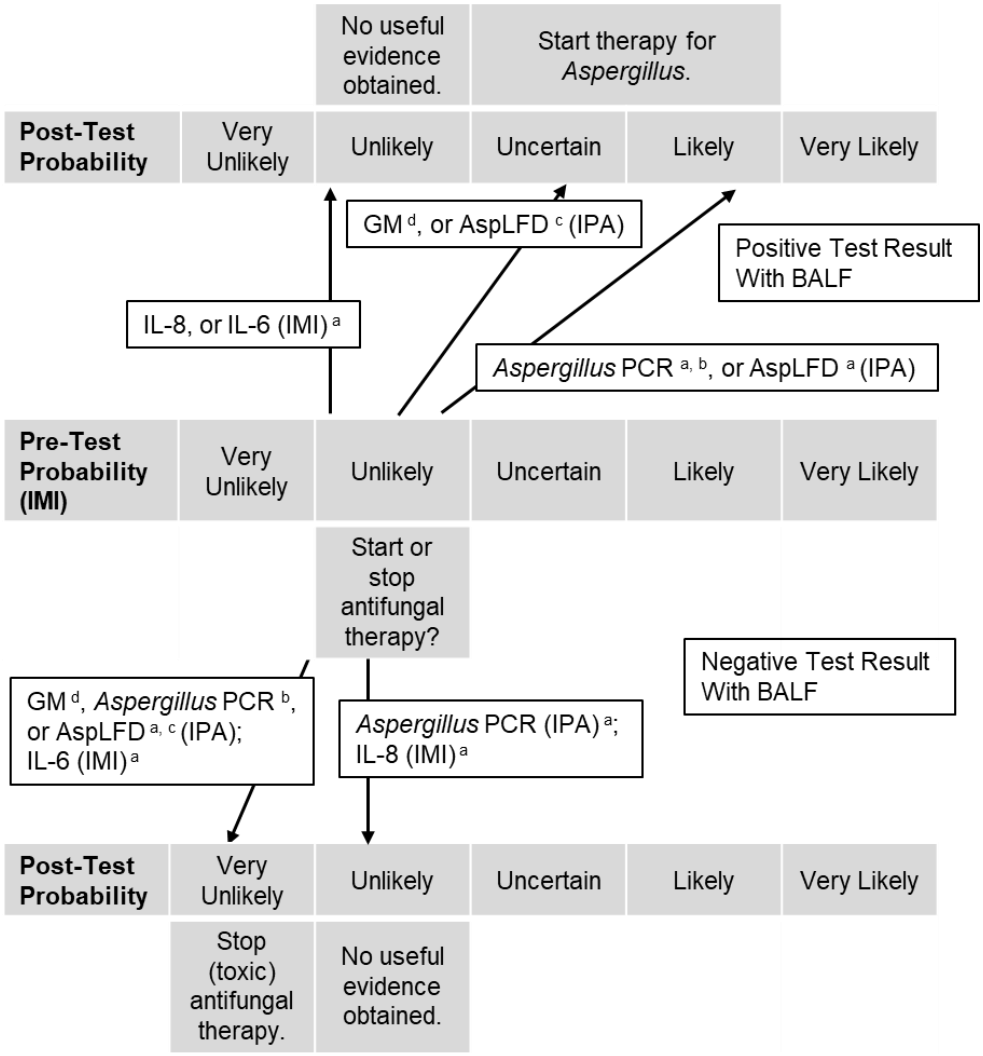


Note. This chart illustrates the influence of a single test result with bronchoalveolar lavage fluid (BALF) on a high pre-test probability for an invasive mold infection (IMI) and on the decision whether an existing mold-active antifungal agent needs to be changed. The abbreviations in brackets indicate the differential diagnosis for which the post-test probability has been changed by the test. Categories for probabilities were adopted from Medow & Lucey (319) (i.e., unlikely pre-test probability = 80%; post-test probabilities: unlikely = 10%–33%, uncertain = 34%–66%, likely = 67%–90%, very likely = 90%–100%). AspLFD = *Aspergillus* Lateral-Flow Device; GM = galactomannan; IL = interleukin; IPA = invasive pulmonary aspergillosis; PCR = polymerase chain reaction.

[a] Likelihood ratios (LR) are based on data from this thesis. [b] LRs are based on data from Avni et al. (275). [c] LRs are based on data from Pan et al. (284). [d] LRs are based on data from de Heer et al. (231) (cut-off 0.5 optical density index).

If a CT scan presents changes that indicate pneumonia but that are not characteristic of IMIs, and serum diagnostics have also not indicated an IMI, the pre-test probability of an IMI will be in the unlikely or uncertain range, depending on the individual constellation. The aim of the diagnostics with BALF is to either (a) make an IMI less likely and preferably rule it out for sure in order to discontinue unnecessary empirical antifungal therapies; or (b) to prove that previous tests with serum and the radiologic examination have produced false-negative results and to parallelly identify the actual IMI in order to establish an antifungal therapy that is as targeted as possible (see Figure 72 and Figure 73). For reducing the likelihood of an IMI in general, low IL-6 levels were suitable. However, high IL-6 levels are too unspecific and cannot be implemented to confirm an IMI. IL-8 has only weak potential to increase the probability of an IMI; therefore, it is helpful at most when the pre-test probability is already increased moderately. According to the literature, *Aspergillus* PCR is the best option for both identifying and ruling out an IPA in this situation, but we were unable to confirm this in our study. The AspLFD and galactomannan test performed with BALF cannot prove an IPA despite positive results if the pre-test probability is in the unlikely range. However, they can be implemented to optimize therapy, and negative results can helpfully reduce the probability of an IPA in this situation. Nevertheless, whether *Aspergillus*-specific diagnostics are also meaningful for assessing the presence of an IMI in general depends on the local epidemiologic situation (i.e., whether IA is the predominant cause of IMIs or not); in our cohort, negative results obtained with the *Aspergillus* PCR and the AspLFD were not able to meaningfully reduce the post-test probability of an IMI.

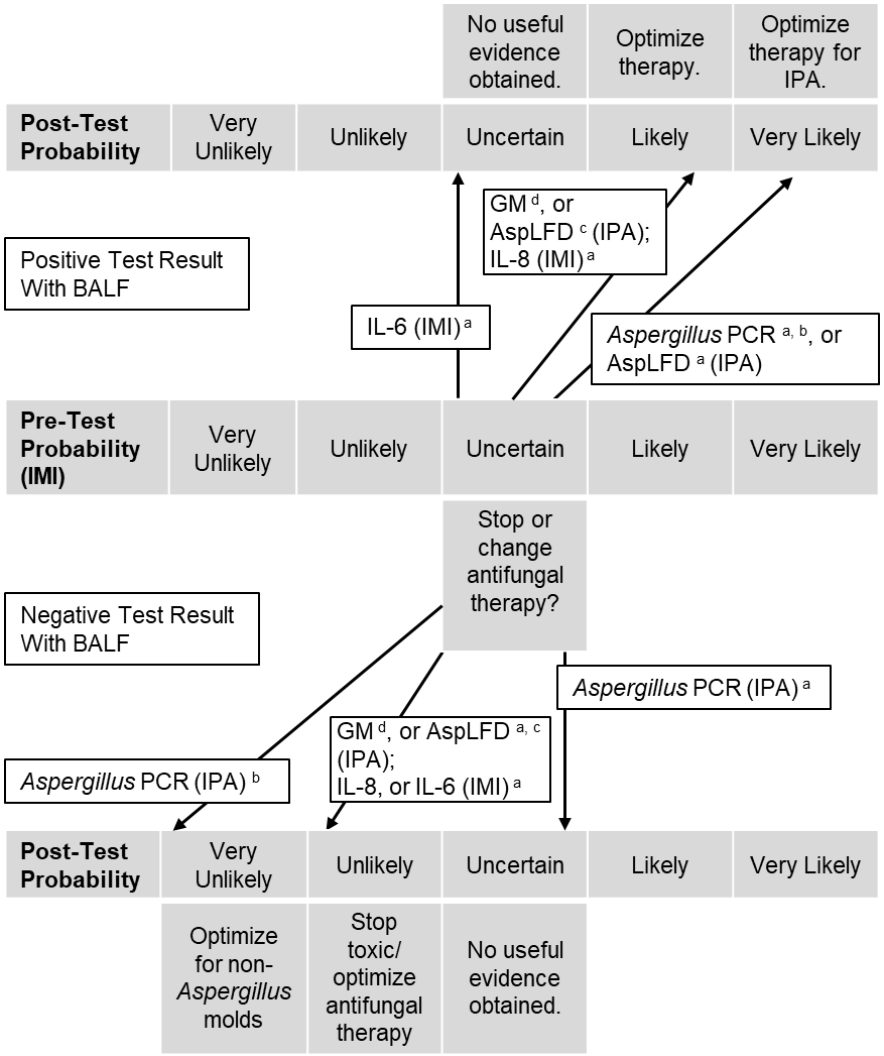
Figure 72 *Impact of Tests With BALF When the Probability of an IMI is Low*



Note. This chart illustrates the influence of a single test result with bronchoalveolar lavage fluid (BALF) on a low pre-test probability for an invasive mold infection (IMI) and on the decision whether a mold-active antifungal agent needs to be started, stopped, or changed. The abbreviations in brackets indicate the differential diagnosis for which the post-test probability has been changed by the test. Categories for probabilities were adopted from Medow & Lucey (319) (i.e., unlikely pre-test probability = 20%; post-test probabilities: very unlikely = 0%–9%, unlikely = 10%–33%, uncertain = 34%–66%, likely = 67%–90%). AspLFD = *Aspergillus* Lateral-Flow Device; GM = galactomannan; IL = interleukin; IPA = invasive pulmonary aspergillosis; PCR = polymerase chain reaction.

[a] Likelihood ratios (LR) are based on data from this thesis. [b] LRs are based on data from Avni et al. (275). [c] LRs are based on data from Pan et al. (284). [d] LRs are based on data from de Heer et al. (231) (cut-off 0.5 optical density index).

Figure 73 *Impact of Tests With BALF When the Probability of an IMI is Moderate*



Note. This chart illustrates the influence of a single test result with bronchoalveolar lavage fluid (BALF) on a moderate pre-test probability for an invasive mold infection (IMI) and on the decision whether a mold-active antifungal agent can be stopped or needs to be changed. The abbreviations in brackets indicate the differential diagnosis for which the post-test probability has been changed by the test. Categories for probabilities were adopted from Medow & Lucey (319) (i.e., uncertain pre-test probability = 50%; post-test probabilities: very unlikely = 0%–9%, unlikely = 10%–33%, uncertain = 34%–66%, likely = 67%–90%, very likely = 90%–100%). AspLFD = *Aspergillus* Lateral-Flow Device; GM = galactomannan; IL = interleukin; IPA = invasive pulmonary aspergillosis; PCR = polymerase chain reaction. [a] Likelihood ratios (LR) are based on data from this thesis. [b] LRs are based on data from Avni et al. (275). [c] LRs are based on data from Pan et al. (284). [d] LRs are based on data from de Heer et al. (231) (cut-off 0.5 optical density index).

Examples of Possible Impact by Interleukin 6 and 8

A possible benefit for selected cases of this study cohort is evaluated in the following (see Table 43). On the one hand, cases were selected that were classified as probable/proven IMI ultimately but had not yet received mold-active antifungal therapy on the day of bronchoscopy. These patients could benefit from improved diagnostics with earlier initiation of therapy. This situation applied to two cases (2/19, 11%) of our probable/proven IMI cases. On the other hand, cases that were classified as unlikely IMI ultimately but received therapy with amphotericin B at the time of bronchoscopy were also selected. This constellation occurred in 13% (8/63) of our unlikely IMI cases. These cases could benefit from diagnostics that allow early exclusion of IMI to prevent side effects from unnecessarily escalated antifungal therapies. Although voriconazole, posaconazole, and caspofungin were also administered in unlikely IMI cases on the day of bronchoscopy, these substances can also be intended as prophylaxis, but unfortunately it was retrospectively not possible to clearly distinguish prophylactic from therapeutic intention by our study data in many cases. Amphotericin B, on the other hand, is administered only with therapeutic intention.

For the probable IPA case in our cohort that did not receive an antifungal therapy on the day of the bronchoscopy, both IL-6 and IL-8 in serum were above our suggested cut-offs and thus indicating an IMI (see Table 43). But so did galactomannan from serum and BALF, and galactomannan measurements have already been established in clinical routine. As IL-8 and IL-6 exhibited poorer overall performances in comparison to galactomannan, adding them to the diagnostic work-up would not have had an additional benefit for management of this probable IPA case.

The probable IMI case that did not receive an antifungal therapy on the day of bronchoscopy was assigned to the probable instead of the possible IM group because of a single highly positive BDG test; however, as an antifungal therapy was not initiated by the attending physicians before BDG testing, it can be assumed that the overall clinical constellation imposed as an unlikely IMI. Nevertheless, the positive LR of BDG is poor and BDG would not be able to confirm an IMI; however, it would increase the probability high enough to initiate antifungal therapy. In contrast, particularly IL-6 in BALF would imply that the BDG test result was false-positive; thus, following the results of IL-6 and IL-8, a mold-active antifungal therapy would not be initiated.

Table 43 *Hypothetical Impact of IL-6 and IL-8 on Antifungal Treatment in Selected Cases*

2008 EORTC/MSG classification	IL-8		IL-6	Change in post-test probability after testing IL-8 and IL-6	Likely impact of IL-8/IL-6 results on mold-active therapy [e]
	Serum [b]	BALF [c]	BALF [d]		
Probable IPA	+	+	+	↑: Very likely IPA	None
Probable IMI	–	–	–	↓: Very unlikely IMI	Maintain (= not start; IL-6 BALF)
Unlikely IMI #1	–	+	+	↑: Uncertain IMI	Maintain (IL-8 BALF)
Unlikely IMI #2	+	–	+	↑: Uncertain IMI	Maintain (IL-8 serum)
Unlikely IMI #3	–	–	+	No change	None
Unlikely IMI #4	–	–	–	↓: Very unlikely IMI	Stop (IL-6 BALF)
Unlikely IMI #5	–	–	+	No change	None
Unlikely IMI #6	–	+	+	No change	None
Unlikely IMI #7	–	+	+	↑: Uncertain IMI	Maintain (IL-8 BALF)
Unlikely IMI #8	–	–	–	No change	None

2008 EORTC/MSG classification	Treatment on day of bronchoscopy	GM (≥ 0.5 ODI)		BDG	Other microbiological evidence [a]	Presumed clinical pre-test probability before testing IL-8/IL-6
		Serum	BALF	Serum		
Probable IPA	None	+	+	–	None	Likely IPA
Probable IMI	None	–	–	+	*Enterobacter sp.*, *Escherichia sp.*, *Klebsiella sp.*	Uncertain IMI
Unlikely IMI #1	Amphotericin B, micafungin	–	–	–	None	Unlikely IMI
Unlikely IMI #2	Amphotericin B	–	–	–	EBV	Unlikely IMI
Unlikely IMI #3	Amphotericin B	–	–	–	None	Unlikely IMI
Unlikely IMI #4	Amphotericin B	–	–	–	None	Unlikely IMI
Unlikely IMI #5	Amphotericin B	–	+	+	Influenza A, EBV	Likely IPA
Unlikely IMI #6	Amphotericin B	–	–	+	None	Likely IMI
Unlikely IMI #7	Amphotericin B	–	–	–	Bocavirus	Unlikely IMI
Unlikely IMI #8	Amphotericin B	–	–	–	*Pneumocystis jirovecii* (PCR, BALF)	Very unlikely IMI

Note. Those cases of the study cohort were selected for which the attending physicians considered either (a) to have no indication for antifungal therapy, but the case was ultimately defined as probable/proven invasive mold infection (IMI) according to the 2008 European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC)/National Institute of Allergy and Infectious Diseases Mycoses Study Group

(MSG) definition; or (b) to have an indication for therapeutic amphotericin B therapy, but the case could only be classified as an unlikely IMI according to the 2008 EORTC/MSG definitions ultimately. The clinical pre-test probabilities assumed in this table before incorporating galactomannan (GM), 1,3-β-D-glucan (BDG), and microbiological test results were defined as 20% for the two probable IMI cases and as 50% for the unlikely IMI cases, reflecting the assessment of the attending physicians whether a therapeutic antifungal regimen was necessary before mycological evidence was available. Likelihood ratios (LR) calculated from meta-analyses for GM (226, 231) and BDG (253) (see also Table 42) were utilized to estimate the clinical probability of IMIs, before applying LRs of interleukin (IL) 8 and IL-6 to further assess their potential impact on disease probability and thereby on management of antifungal therapies. The qualitative categorization of post-test probabilities was adopted from Medow and Lucey (330): very unlikely = 0%–9%, unlikely = 10%–33%, uncertain = 34%–66%, likely = 67%–90%, and very likely = 91%–100%. Possible consequences following the IL-6 and IL-8 results included starting, stopping, or maintaining antifungal therapy.
+ = positive; – = negative; BALF = bronchoalveolar lavage fluid; EBV = Epstein-Barr virus; IPA = invasive pulmonary aspergillosis; ODI = optical density index; PCR = polymerase chain reaction.
[a] From blood or respiratory samples. [b] ≥ 55.6 pg/mL. [c] ≥ 877.7 pg/mL. [d] ≥ 55.7 pg/mL.
[e] Information in brackets indicates the test and sample type that had the largest impact on a changing post-test probability.

However, it is acknowledged that it is possible that this case is falsely classified into the probable/proven IMI group, especially as the 2020 EORTC-IDG/ MSGERC definitions would have assigned it into the possible IMI group; but changing interpretation of the reference test case-by-case after knowledge of the index test results would introduce bias. Thus, it must be admitted that IL-6 and IL-8 results were false-negative in this case, hindering the introduction of a mold-active antifungal therapy in this uncertain situation.

Differing results of IL-8 in serum and BALF were observed within four unlikely IMI cases with amphotericin B therapy (i.e., unlikely IMIs number 1, 2, 6, and 7 in Table 43). IL-8 in serum has the better overall performance, and a positive IL-8 test with serum will outweigh a negative IL-8 in BALF and will still increase the post-test probability; similarly, a negative IL-8 test with serum will also outweigh most of the effect of a positive IL-8 in BALF, keeping

the post-test probability at nearly the same level as the pre-test probability. As an antifungal therapy was initiated by the attending physicians before testing galactomannan and BDG, it can be assumed that the overall clinical constellation imposed at least as an uncertain IMI. With negative galactomannan and BDG tests, an IA would be very unlikely; however, a non-*Aspergillus* IMI is not necessarily excluded, and a combination of false-positive IL-8 and IL-6 tests with BALF will increase the post-test probability again, indicating that a possibly unnecessary mold-active antifungal therapy should not be stopped (i.e., unlikely IMIs number 1 and 7 in Table 43). Similarly, IL-8 in serum has a good positive LR, therefore, a false-positive result in combination with a false-positive IL-6 in BALF will have the same impact on post-test probability (i.e., unlikely IMI number 2 in Table 43). Even if the post-test probability does not increase to that extent, at least the uncertainty within clinical case management will increase. In case of a false-positive BDG test, however, a combination of negative IL-8 in serum and false-positive IL-8 and IL-6 in BALF at least would not increase the post-test probability in a relevant way (i.e., unlikely IMI number 6 in Table 43).

IL-6 has the most effective negative LR compared to IL-8; however, a negative IL-8 in serum and BALF combined with a positive IL-6 in BALF will still lower the pre-test probability. Nevertheless, when starting from an uncertain pre-test probability, the resulting effect is limited and likely not decisive after receiving negative galactomannan and BDG result (i.e., unlikely IMI number 3 in Table 43). Additionally, in situations with a false-positive galactomannan and/or BDG test, negative IL-8 tests will also not be enough to relativize the false-positive tests (i.e., unlikely IMI number 5 in Table 43).

Nevertheless, if IL-8 and IL-6 in both serum and BALF are negative, they can diminish the last doubts about stopping mold-active antifungal therapies in cases with negative galactomannan and BDG (i.e., unlikely IMI number 4 in Table 43). However, if there is already a very unlikely pre-test probability for IMIs because another non-mold fungus has been identified (e.g., *Pneumocystis jirovecii*), there will be no relevant additional effect on antifungal therapeutic management by IL-6 and IL-8 (i.e., unlikely IMI number 8 in Table 43). In summary, in both of our two probable IMI cases without mold-active antifungal therapy, IL-6 and IL-8 would not have been helpful. They would have had no decisive effect on the therapeutic management despite being true-positive, or they would have deferred the initiation of a possibly necessary mold-active antifungal therapy by resulting false-negative. In 13% (1/8) of unlikely IMI cases with amphotericin B therapy, particularly IL-6 in BALF

would have been able to lower the post-test probability so that a probably unnecessary therapy could have been stopped without risk. In 25% (2/8) unlikely IMI cases, there would have been no helpful impact by IL-6 and IL-8; in further 25% (2/8) of unlikely IMI cases, IL-6 and IL-8 would not have been able to relativize false-positive galactomannan and BDG results. In the remaining 38% (3/8) of unlikely IMI cases with amphotericin B therapy, false-positive IL-6 and/or IL-8 results would have implied a further need for a probably unnecessary mold-active antifungal therapy. Of course, these considerations entail several variables that contain great uncertainties and are not generalizable. However, these examples suggest that the high frequency of false results by IL-6 and IL-8 can be problematic for the therapeutic management if they are incorporated generally and imprudently in IMI diagnostics, even in combination with other diagnostic tests that perform well. In the end of the search for immunologic biomarkers in IMIs, there will likely not be a bundle of several biomarkers used by default in every diagnostic work-up but likely a well-selected assortment, consisting of one or two markers with the best performance that each are intended for an individual specific question. Furthermore, the 2008 EORTC/MSG and 2020 EORTC-IDG/MSGERC definitions seem to be imperfect in a relevant extent, as discrepancies to clinical disease probabilities are not uncommon; therefore, better possibilities for verifying the true condition of cases in diagnostic studies are needed. Until then, it will be one of the most important points for diagnostic studies, especially regarding non-*Aspergillus* IMIs, to achieve histopathological verifications of the true case conditions, either by sterile samples and biopsies while the patient is alive or by autopsy if the patient deceases.

Longitudinal Evaluation of Cytokines and Chemokines

It was assumed that the duration for which plasma samples are stored at room temperature prior to centrifugation in routine clinical practice at our center would be less than 4 hours. Particularly, it was also expected that the concentrations would remain stable after centrifugation when stored at +4 °C up to a maximum of 4 days (334, 335). Thus, the concentrations of biomarkers in plasma should have remained stable despite non-standardized processing. At least, no consistently elevated concentrations were measured, as would have been expected if storage at room temperature prior to centrifugation had been too long. The factors that led to the discrepancy between the serum and plasma samples remained unclear ultimately. In any case, no noticeable changes in concentration were found during 4 days for IFN-γ, IL-10, and sIL-2R; these biomarkers were ultimately accepted as reliable. We

observed changing concentrations over time only for CCL5 in possible IMI cases. However, as demonstrated, the underlying measurements are questionable in terms of reliability. Nevertheless, particularly the observation that probable/proven IMIs did not regenerate CCL5 levels is consistent with a publication of Ellis et al. (91) who made similar observations for patients that died of IFIs. In addition, there were in vitro experiments that demonstrated the inhibition of CCL5 secretions by molds (77, 90). In the case of confirmation by further studies, this failing regeneration of CCL5 levels in probable/proven IMIs could be implemented for diagnostic or risk stratification purposes.

Associations of Cytokines and Chemokines With Mortality

Associations between concentrations of IL-6 and IL-8 with mortality had already been known and were also evident in our data, namely, for IL-6 with both BALF and serum and for IL-8 with serum. Since IMIs are also associated with high mortality, a simultaneous association of IL-6 and IL-8 with both factors is not surprising. When looking on probable/proven IMI cases only, however, lower IL-22 concentrations in BALF were associated with death within 90 days, but not concentrations of IL-6 or IL-8. This is interesting, as the effect size was quite high and a correlation between exacerbations of IPA and blocking of IL-22 has been observed in preclinical studies in mice (160).

Other Factors Possibly Influencing Biomarker Concentrations

One difficulty in assessing the diagnostic usefulness of cytokines and chemokines is their lack of specificity. They are not secreted exclusively by, for example, a single cell line, and not only in response to a sole specific stimulus. This means that there are a multitude of possible influencing factors. In our study, common influencing factors were particularly underlying malignant diseases affecting the cellular immune defense, concurrent infections by other microorganisms, and medications comprising the immune response.

Hematological malignancies themselves can be associated with changes in the cytokine and chemokine milieu (93). In our study, IL-8 levels in serum and IL-6 levels in BALF were indeed slightly different in cases with ALL than in cases with other underlying diseases. IL-6 has been associated with tumor activity in several malignancies, and an association of B-cell ALL with elevated serum IL-6 levels has been observed in children (336, 337). However, we were only able to demonstrate an association of IL-6 and ALL in BALF. This may likely represent pulmonary infectious processes associated with ALL.

Nevertheless, there are studies reporting associations of IL-8 concentrations in serum and ALL; however, IL-8 was also reported to be associated with bacterial infections at the same time (338, 339). In addition, we did not observe higher IL-8 levels in the serum of our probable/proven IMIs with ALL than in the serum of probable/proven IMIs with other underlying diseases. At the same time, the cases with ALL as the underlying disease were not notably more frequent in the probable/proven IMI group than in the unlikely/possible IMI group. In cases with AML, the concentrations of CCL5 in BALF were lower than in cases with other underlying diseases. However, this difference could not be detected in serum, and the influence of AML on CCL5 concentrations has been primarily noted in the form of increased concentrations (340). In conclusion, it is considered unlikely that our findings for IL-8, IL-6, and CCL5 were directly influenced to a relevant amount specifically by AMLs, NHLs, or ALLs. However, we observed generally low levels particularly for IFN-γ, IL-17A, IL-4, and TNF-α, which may be the consequence of the generally immunocompromised status of our cases.

It is known that bacterial infections affect IL-8 and IL-6 concentrations (84, 131). However, a post-hoc analysis of our data did not reveal any differences in biomarker concentrations between cases with and without positive bacterial cultures. However, viral infections seemed to moderately increase our concentrations of IL-8 in the BALF (see Figure 68). At the same time, proportionately more cases with positive tests for viruses accumulated in the probable/proven IMI group in comparison to the unlikely/possible IMI group (see Table 17). Associations between viral infections and elevated IL-8 concentrations in respiratory secretions are known for rhinoviruses, respiratory syncytial virus (RSV), influenza, and parainfluenza, among others, and increased concentrations in plasma were reported in association with herpes virus detection (341-346). In some of these studies, IL-6 is also mentioned as being elevated in connection with viral infections, but no demonstrable correlation was found in our data (341, 343-345). In addition, we could not prove that levels of IL-8 in serum and IL-17A in BALF were associated with a positive viral finding. Furthermore, we have already demonstrated in our previous publication with a case-control design, in which evidence of viruses was one criterion within the matching process, that IL-6 and IL-8 in serum and BALF retained their diagnostic potential despite possible concurrent viral infections (81).

Further post-hoc analysis did not demonstrate any evidence that glucocorticoids have had an inhibitory effect on IL-8 concentrations in serum and BALF or IL-6 in BALF. Cases receiving T cell suppressants were unevenly distributed among the IMI probability groups, but there were no differences in IL-6 and IL-17A concentrations in BALF or IL-8 concentrations in BALF and serum between cases receiving T cell suppressants and other cases. Low leukocyte counts are another hallmark of a compromised immunity and may have affected levels of interleukins. Indeed, we observed correlations between low leukocyte counts and IL-6 and IL-8, but these were inverse and therefore most likely a consequence of a higher probability of infections due to deficient cellular immunity than a direct interdependency between leukocyte counts and interleukins.

There was no sign of a negative influence of mold-active antifungal therapy on the diagnostic performance of IL-6 in BALF and IL8 in serum and BALF: The diagnostic test performances of IL-6 and IL-8 reported in this thesis did not deteriorate when only cases receiving mold-active antifungals longer than 1 day were respected in analysis.

After the patient recruitment of this work had already been completed, Donelly et al. (127) published a revised version of the EORTC/MSG criteria in 2020. In particular, the exclusion of BDG from the mycological criteria would influence the composition of our case categorization and thus also the results. Whether the acceptance of consecutive positive *Aspergillus* PCRs from blood/serum as a mycological criterion would have had an influence that would have compensated for the exclusion of the BDG cannot be assessed, since routine *Aspergillus* PCRs had not been established at the time of patient recruitment. A post-hoc reclassification of cases using 2020 EORTC-IDG/MSGERC definitions would have been susceptible to bias, therefore the analysis of this work was still performed according to the 2008 EORTC/MSG definitions, as this was intended in the study protocol and was state of the art at the time of patient recruitment.

Limitations and Strengths

An important limiting factor in our study results is the low number of probable and especially of proven IMI cases. This is due to the low prevalence of IMIs, even in high-risk populations. The two systematic reviews published by Cruciani et al. (274) and Leeflang et al. (226) gave point estimates of the prevalence of IA that were calculated from the included diagnostic studies. They both reported a median study prevalence of 11% for IA (226, 274). Cruciani et al. also calculated that rough prevalences of IA were about 40% lower in patients

with mold-active prophylaxis than in patients without mold-active prophylaxis (i.e., 10% vs. 17%, respectively) (274). Regarding non-*Aspergillus* IMIs, incidences of less than 1% were commonly observed, but local epidemiology may differ (10, 11). Our study prevalences were 10% (11/106) for probable IPAs and 8% (8/106) for probable/proven non-*Aspergillus* IMIs, (or 3% [3/106] for probable/proven non-*Aspergillus* IMIs if BDG had not been accepted as a mycological criterion for defining IMIs). Thus, the number of cases with IA or IMI is comparable to similar studies. The variability of our measured data, however, was generally high, which led to very broad 95% CIs. To obtain 95% CIs whose lower limits would not only indicate significance but also diagnostic relevance, subsequent studies should aim to halve these 95% CIs at least. But this would require an approximately 4-fold rise in the number of cases (300). Thus, a corresponding extension of the recruitment period or an expansion of patient recruitment to multiple study centers will likely be necessary for more precise estimations of diagnostic performances of immunological biomarkers in future studies.

In comparison to proven IMIs, probable IMI cases bear the uncertainty that they may have arisen from false-positive results of the mycological criteria of the EORTC/MSG definitions, thus potentially skewing the results. Increasing the rate of proven IMIs compared to probable IMIs would require more invasive diagnostic procedures. However, the possibility of utilizing invasive diagnostics is usually at its limit because of the risks for the patient. A higher rate of proven rather than probable IMIs will likely require more autopsies. However, experience indicates that obtaining informed consent for an autopsy for study purposes will lead to more patients refusing study participation.

Concentrations of cytokines and chemokines are influenced by many factors. Other concurrent infections, underlying diseases, or differently impaired conditions of the immune system itself could have influenced our results (see also chapter Other Factors Possibly Influencing Biomarker Concentrations above). Particularly, the impaired immune response could be the reason for many low biomarker concentrations despite active infections: On the one hand, other studies that investigated immunological biomarker concentrations in IA or chronic pulmonary aspergillosis but that mainly recruited non-hematological patients observed concentrations of biomarkers that would actually have been detectable with our immunoassay (92, 323, 324, 347). On the other hand, our concentrations of biomarkers that clustered below the calibrated measurement ranges of our immunoassay were largely comparable to those concentrations measured by Goncalves et al. (83). However, Goncalves

et al. seemed to use an assay that was more suitable for low biomarker concentrations. Nevertheless, low concentrations in BALF may also be a problem of standardizing BAL techniques; dilutive effects could also have impacted our results.

Because of the amount of possibly influencing factors, our results must be verified and confirmed in further studies with other cohorts. However, case-control studies matching various known relevant co-factors have already demonstrated diagnostic potential for IL-6 and IL-8 to detect IPAs (81, 83). Nevertheless, our cohort study design is better suited than a matched case-control study to represent the problematic diagnostic situation in real life. Particularly one-time measurements on the same day make it possible to directly compare the diagnostic performances of the individual methods with each other. In addition, our results can be applied to populations with a high proportion of mold-active prophylaxis or therapy in which diagnostics using fungal antigen or fungal DNA based methods are challenging.

Another problem of studies about cytokines and chemokines that limits validity of study results arises when sample processing is not sufficiently standardized. This seemed to have affected our evaluation of the plasma samples: Ultimately, only three out of our 11 biomarkers yielded measurements with plasma samples that demonstrated acceptable congruence with the standardized evaluation of serum samples. Also involving standardization issues, the characteristics of our *Aspergillus* PCR assay and DNA extraction method that may have had an impact on our results have been already explained above (see chapter Diagnostic Performances). It is likely that better performance values (particularly sensitivity) will be achieved when implementing the standardization recommendations of the FPCRI.

The group of possible diagnostically useful biomarkers is large and by no means fully covered by our selection. It should be emphasized that not all key cytokines and chemokines involved in mold defense are represented in our selection of immunological biomarkers. Other chemokines that were elevated in studies with human cells in connection with *Aspergillus spp.* are CXCL2, CCL2, CCL3, CCL4, and CCL20 (77, 79). In the context of an effective cytokine response to IA, elevations of IL-1/IL-1β, IL-2, IL-12, IL-23, and TGF-β were recognized (38, 41, 77, 105). Out of these examples, Goncalves et al. (83) have already demonstrated a diagnostic potential in IPAs for IL-1β and IL-23 in the BALF. Regarding soluble effector molecules, particularly pentraxin 3 and mannose-binding lectin are discussed as possible future biomarkers (52). Moreover, additional diagnostic approaches that are

currently under evaluation include LFDs for non-*Aspergillus* molds, an ELISA for fungal α-1,6-mannan epitopes, and measurements of volatile fungal metabolites from the breath (348-353) .

Conclusion

In summary, our results on IL-8 in serum and BALF and IL-6 in BALF do not contradict but strengthen previously published findings. Regarding IMIs, these two markers may be able to decrease post-test probabilities via low concentrations of IL-6 and IL-8 in BALF or to increase post-test probabilities via high concentrations of IL-8 in serum. However, established *Aspergillus*-specific tests likely offer better diagnostic performances for diagnosing IAs. In BALF, the AspLFD provided better overall performance than IL-6, IL-8, and the *Aspergillus* PCR. In serum, however, IL-8 was superior to the AspLFD. Notably, IL-8 in serum also performed better than BDG for diagnosing IPA. Nevertheless, IL-6 and IL-8 may be most useful for non-*Aspergillus* IMIs which diagnostic verifications are still most challenging. Noticeably, concentrations of some other biomarkers (particularly IL-17A, IL-10, CCL5, IFN-γ, and TNF-α) were generally low; causalities remained unclear but this observation may be connected to the immunocompromised state of the study population. Nevertheless, differences in probability distributions of IL-17A concentrations were observed in BALF between unlikely and probable/proven IMIs but without a clear discriminative potential. As expected, high IL-6 and IL-8 concentrations were associated with overall mortality. Interestingly, low IL-22 concentrations in BALF exhibited an association with mortality specifically in probable/proven IMIs.

Our study was designed exploratively but not confirmatively; therefore, confirmation of our results is necessary through further studies. To confirm not only the statistical significance but also a diagnostic relevance of our results, future studies should aim for an approximately 4-fold higher study size to narrow 95% CIs; additionally, power analysis should respect small effect sizes. Sample collection processes need to be tested beforehand to confirm reliability if samples are collected in clinical routine. To reduce uncertainties with the reference standard, autopsies should be implemented for all deceased patients. Apart from IL-6 and IL-8, 95% CIs of our ROC analysis indicated that a relevant predictive potential would basically be possible for IL-10 and CCL5 in serum, and IL-15, IL-17A, and IL-22 in BALF.

If our results are confirmed, particularly IL-6 in BALF and IL-8 in serum will be a step forward in filling the gap in diagnostics for non-*Aspergillus* IMIs, although they will not help in identifying the causative fungal genera and will have issues with sensitivity and specificity, but they have the potential to perform better than BDG.

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Appendix

Materials

Sample vessels

- serum
    - **collected in:** Vacuette Tube CAT Serum, with separating gel and clot activator (clot activator main component: silicon dioxide particles). Greiner Bio-One GmbH, Kremsmünster, Austria.
    - **stored in:** Eppendorf Safe-Lock Tubes. Eppendorf SE, Hamburg, Germany.
- whole blood
    - **collected and shipped in:** Vacuette Tube K3E K3EDTA, containing tripotassium EDTA. Greiner Bio-One GmbH, Kremsmünster, Austria.
- plasma
    - **collected in:** Vacuette Tube LH, with lithium-heparin and separating gel; Greiner Bio-One GmbH, Kremsmünster, Austria.
    - **stored in:** Eppendorf Safe-Lock Tubes. Eppendorf SE, Hamburg, Germany.
- BALF
    - **collected in:** common sterile sample beakers (i.e., uncoated and without additives), no pretreatment.
    - **stored in:** Thermo Scientific Nunc Biobanking and Cell Culture Cryogenic Tubes. Thermo Fisher Scientific Inc., Waltham (MA), United States.

LOT of AspLFDs: 11215-028-2. Distributed by OLM Medical Ltd., Newcastle Upon Tyne, United Kingdom.

Sample Collection Process

Serum and Whole Blood Samples

Samples are taken by the investigator after the patient has given informed consent to participate in the study.

- Preferably collect blood samples via separate peripheral venipuncture. If the patient only consents to collection from an existing central venous catheter: Flush the catheter with 0.9% (wt/vol) sodium chloride solution. Then discard at least 10 mL of sampled blood before collecting study samples.
- Immediately transport samples to the microbiological laboratory.
- serum samples, further processing
    - Centrifuge at 4,000 rpm for 15 minutes.
    - Aliquot supernatant into tubes for storage.
    - Freeze at –20 °C until all study samples from the patient have been collected.
    - Store at –70 °C until further use.
- whole blood samples, further processing
    - Store at +4 °C in a refrigerator.
    - Arrange for refrigerated overnight transport to the University Hospital Mannheim, Mannheim, Germany, for *Aspergillus* PCR testing.

Plasma Samples

Plasma samples are taken by nursing staff as part of routine clinical care. The samples from the ward are gathered at room temperature and then are transported to the central laboratory by the hospital courier service. Direct processing of samples in the central laboratory. Sample residues are stored at +4 °C for 4 days by default.

- On the day of bronchoscopy, contact the central laboratory and have the routinely stored plasma samples reserved.
- Aliquot supernatants into tubes for storage.
- Freeze at –20 °C until all study samples from the patient have been collected.
- Check daily for up to 4 days after bronchoscopy to see if any further plasma samples have arrived at the central laboratory. Have the remaining samples reserved, aliquot them, and store them at –20 °C until all study samples from the patient are complete.
- Store at –70 °C until further use.

Bronchoalveolar Lavage Fluid

BALF is delivered by the bronchoscopy unit to the microbiology laboratory immediately after collection. There, the amount required for the requested tests is withdrawn. The residue is stored at +4 °C until aliquoting by the investigator on the same working day.

- Mix thoroughly using sample tube shaker.
- Aliquot BALF into tubes for storage.
- Request refrigerated overnight shipping to University Hospital Mannheim, Mannheim, Germany, for the *Aspergillus* PCR, pickup on the same day. For this purpose, keep one aliquot at +4 °C until pickup.
- Store the remaining aliquots at –20 °C until all samples have been collected from the patient.
- Store at –70 °C until further use.

Deoxyribonucleic Acid Extraction Method

For the *Aspergillus* PCR, the erythrocytes in the EDTA whole blood sample were first lysed, then the sample was centrifuged, the supernatant discarded, and the remaining leukocyte-containing sediment washed, as described by Skladny et al. (296):

> A total of 3 to 5 ml of peripheral blood was mixed with 5 volumes of erythrocyte lysis buffer (0.155 M NH_4Cl, 0.01 M NH_4HCO_3, 0.1 mM EDTA [pH 7.4]), and the mixture was incubated for 10 min at 4°C. After lysis of erythrocytes, the sample was centrifuged at 300 × *g* for 10 min. The supernatant was discarded, and the leukocytes were washed once with 1× phosphate-buffered saline (10× phosphate-buffered saline is 1.4 M NaCl, 50 mM KCl, 90 mM $Na_2PO_4 \cdot 2H_2O$, and 20 mM KH_2PO_4 [pH 7.4]) and recentrifuged. (p. 3866)[1]

BALF samples were first centrifuged before the supernatant had been discarded. The remaining sediments from the EDTA whole blood sample and the BALF sample were further processed as described by Skladny et al. (296):

> BAL samples were transferred to 1.5-ml tubes, the tubes were centrifuged at 13,000 rpm for 5 min (bench minifuge; Heraeus, and the supernatant was removed. Sedimented cell material from both blood and BAL specimens was processed as follows: the leukocyte pellet was resuspended in 300 μl of 1× phosphate-buffered saline and the mixture was incubated with 100 to 125 U of lyticase (50,000 U; Sigma) for 30 min at 37°C to achieve degradation of fungal cells. Residual human and fungal cell material was treated with 500 to 1,000 μg of proteinase K (Boehringer Mannheim, Mannheim, Germany) and 0.5% sodium dodecyl sulfate (Sigma) at 55°C for 1 h. Residual cell material was then lysed by incubation with an additional 100 μl of 2× *Aspergillus* extraction buffer (400 mM Tris-Cl, 1 M NaCl, 20 mM EDTA, 2% sodium dodecyl sulfate) for 30 min at 65°C. The purification of fungal and human DNA was performed by conventional phenol-chloroform extraction [Sambrook et al. (297)]. The DNA was precipitated by the addition of 0.7 volume of

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[1] From "Specific Detection of *Aspergillus* Species in Blood and Bronchoalveolar Lavage Samples of Immunocompromised Patients by Two-Step PCR," by H. Skladny, D. Buchheidt, C. Baust, F. Krieg-Schneider, W. Seifarth, C. Leib-Mösch, and R. Hehlmann, 1999, *Journal of Clinical Microbiology, 37*(12), pp. 3865–3871 (https://doi.org/10.1128/jcm.37.12.3865-3871.1999)(296). No modifications were made. Copyright 1999 by American Society for Microbiology. Reprinted with permission.

isopropanol, pelleted, and washed once with 70% ethanol and air dried. The DNA concentration was assessed by spectrophotometry at 260 and 280 nm. (p. 3866; for copyright attribution, see Footnote 1).

Execution of the *Aspergillus*-Specific Lateral Flow Device Assay

The following laboratory protocols were prepared based on the instructions for use provided with the AspLFD (298).

Serum and Hemorrhagic Bronchoalveolar Lavage Fluid Samples

1. Thaw sample and mix it thoroughly using a sample tube shaker.
2. Centrifuge at 14,000 rpm for 1 min.
3. Take 50 µL sample supernatant and mix it thoroughly with 100 µL buffer (provided by manufacturer) in an Eppendorf Safe-Lock Tube, using a sample tube shaker.
4. Heat it for 3 min in a water bath at 100 °C.
5. Centrifuge at 14,000 rpm for 5 min.
6. Take 100 µL supernatant and apply it to the AspLFD sample well.
7. Read results after 10 min.

Bronchoalveolar Lavage Fluid, Non-Hemorrhagic

1. Thaw sample and mix it thoroughly using a sample tube shaker.
2. Centrifuge at 14,000 rpm for 1 min.
3. Take 100 µL supernatant and apply it to the AspLFD sample well.
4. Read results after 10 min.

Publications With First Authorship Related to This Thesis

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ADVANCES IN DIAGNOSIS OF INVASIVE FUNGAL INFECTIONS (S CHEN, SECTION EDITOR)

Lateral Flow Assays for the Diagnosis of Invasive Aspergillosis: Current Status

Sven Heldt[1] · Martin Hoenigl[1,2,3,4]

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Abstract
Purpose of Review Diagnosis during early stages of invasive aspergillosis (IA) and targeted antifungal treatment has the potential to improve survival significantly. Despite advances in the diagnostic arsenal, invasive mold infections remain difficult to diagnose—especially at early stages before typical radiological signs develop. Varying availability and time-to-results are important limitations of current approved biomarkers and molecular assays for diagnosis of IA. Here, we will give an update on the *Aspergillus*-specific lateral-flow device (LFD) test. We further review promising findings on feasibility of point-of-care (POC) detection of urinary excreted fungal galactomannan-like antigens.
Recent Findings POC LFD assays for detection of *Aspergillus* antigens are currently in development. The *Aspergillus*-specific LFD test, which is based on the JF5 antibody (Ab), detects an extracellular glycoprotein antigen secreted during active growth of *Aspergillus* spp. The test has shown promising results in various studies. In addition, a monoclonal Ab476-based LFD for POC detection of urinary excreted fungal galactomannan-like antigens has been developed but needs further validation.
Summary Important advances have been made in the development of LFD assays for IA. Most promising is the *Aspergillus*-specific LFD test; commercial availability is still pending, however. The search for reliable POC tests for other molds, including mucorales, continues.

Keywords Aspergillus lateral flow device test · Point of care · Galactomannan-like antigens · MAb476 · JF5 · Bronchoalveolar lavage · Urine · Serum · Monoclonal antibody · Invasive aspergillosis

This article part of the Topical Collection on *Advances in Diagnosis of Invasive Fungal Infections*

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Introduction

Invasive aspergillosis (IA) is associated with high mortality rates [1–5]. Early and reliable diagnosis and rapid initiation of appropriate antifungal therapy has been shown to improve survival significantly [6, 7]. Culture-based approaches are important for detection of fungal species and resistance testing; however, they are limited by low sensitivities—in particular during early phases of infection—and long turnaround time [8•]. Significant advances to the field were brought by the introduction of non-cultural diagnostic tests for IA in blood and bronchoalveolar lavage fluid (BALF), including galactomannan antigen (GM) testing [9•, 10 –13], PCR [14••, 15, 16••, 17], and beta-D-glucan (BDG) testing [18–24] in patients at risk [25•, 26, 27, 28•, 29]. In line with the introduction of non-cultural diagnostic tests, the rate of fungal infections diagnosed pre-mortem (versus post-mortem) was shown to increase from 16 to 51% in a large autopsy study [30].

Despite these significant advancements, the availability of these non-cultural diagnostic tests and time-to-results often varies with the size, specialization, and resources of the medical institution. "Pregnancy test-like" point-of-care lateral flow

[2] Copy of "Lateral Flow Assays for the Diagnosis of Invasive Aspergillosis: Current Status," by S. Heldt and M. Hoenigl, 2017, *Current Fungal Infection Reports*, *11*, pp. 45–51 (https://doi.org/10.1007/s12281-017-0275-8)(287). No modifications were made. Copyright 2017 by the authors. This article is an open access publication distributed under the terms of the CC BY 4.0 license (http://creativecommons.org/licenses/by/4.0/).

assays for detection of *Aspergillus* antigens are currently in development and may overcome these limitations.

Aspergillus-Specific Lateral Flow Device Test

The point-of-care *Aspergillus*-specific lateral-flow device test (LFD) uses a mouse monoclonal antibody, JF5, which binds to an extracellular glycoprotein antigen secreted by *Aspergillus* spp. only during active growth [31]. The LFD can be used for testing of serum and BALF samples and shows cross-reaction with *Penicillium* spp. only [31]. While serum samples require heating, centrifugation, and addition of a buffer solution before testing, BALF samples can be tested without any pre-treatment [32]. After 15 min of incubation time, results are read by the naked eye and are interpreted depending on the intensity of the test line as negative (−) or weak (+) to strong (+++) positive and have been shown to be reproducible between laboratories and studies [33, 34].

To date, two studies evaluated the diagnostic performance of the LFD in serum samples from adult patients with hematological malignancies [14, 35•], reporting sensitivities of 40 and 82% and specificities 87 and 80% for probable/proven invasive aspergillosis (IA) according to modified EORTC/MSG criteria [36], respectively. A meta-analysis, which included also data from the LFD development study [31] (i.e., in addition to the two studies mentioned above), reported a pooled sensitivity of 68% (95% confidence interval (CI), 52–81%), specificity of 87% (95% CI, 80–92%), and diagnostic odds ratio (DOR) of 11.90 (95% CI, 3.54–39.96) for differentiating proven/probable versus no IA cases in serum samples [37•]. Another very recently published study ignored the recommendations of the manufacturer by using the LFD in serum samples without pretreatment and found poor performance confirming that pretreatment of serum samples is a necessary step and that recommendations of the manufacturer should be followed [38]. Overall, the requirement for pretreatment has been a major limitation of serum LFD testing, as has been the inconsistency of reported results. Further studies, including multicenter studies, are needed to determine whether LFD serum testing can be recommended for clinical routine.

In contrast to serum testing, BALF LFD testing has been evaluated in a number of studies including multicenter studies and in different patient populations. Results from the first four-part, retrospective-part, prospective studies (including two multicenter studies) which evaluated the LFD in mostly patients with underlying respiratory diseases [39••] and solid organ transplant recipients [28, 40••, 41] but also a smaller proportion of patients with underlying hematological malignancies [28, 41], were summarized in the meta-analysis reporting a pooled sensitivity of 86% (95% CI, 76–93%), specificity of 93% (95% CI, 89–96%), and DOR of 65.94 (95% CI, 27.21–159.81) for IA when using BALF samples [37].

Since then, BALF LFD testing has been evaluated in multicenter studies in intensive care unit patients [42••] and patients with underlying hematological malignancies [43], as well as a number of single-center studies [16••, 44–47]. The up-to-date performance of the LFD in BALF samples for different patient groups as well as the overall performance per sample are depicted in Table 1. Published data indicates that to date, 792 BALF samples have been tested at 6 different medical universities, including 113 samples from patients with probable/proven IA and 552 samples from patients with no evidence of IA, resulting in an overall sensitivity of 73% and specificity of 90% for probable/proven IA versus no evidence for IA. While the overall positive predictive value (PPV) was 61% and the negative predictive value (NPV) 94% in samples tested to date, both PPV and NPV will depend on the prevalence of IA in tested populations as displayed in Fig. 1. For example, in a patient cohort with an IA prevalence of 1%, the PPV will be 7.6%, while the NPV will be 99.7%; the PPV will go up and the NPV down with increase of IA prevalence (e.g., 5% IA prevalence: PPV 28%, NPV 98.4%; 10% IA prevalence: PPV 45%, NPV 96.8%; 20% IA prevalence: PPV 65%, NPV 93%; always assuming 73% sensitivity and 90% specificity).

Table 1 Per BALF sample performance of the BALF *Aspergillus* LFD for probable/proven invasive pulmonary aspergillosis versus no evidence for invasive pulmonary aspergillosis in different patient cohorts (percentage and absolute numbers)[a]

Patient group	Sensitivity	Specificity	PPV	NPV
Overall[b]	73% (83/113)	90% (498/552)	61% (83/137)	94% (498/528)
Solid organ transplantation	94% (15/16)	92% (89/97)	65% (15/23)	99% (89/90)
Intensive care unit	79% (26/33)	85% (176/206)	46% (26/56)	96% (176/183)
Respiratory diseases	78% (25/32)	91% (196/215)	57% (25/44)	97% (196/203)
Hematological malignancies	67% (36/54)	91% (126/139)	73% (36/49)	88% (126/144)

PPV positive predictive value, *NPV* negative predictive value

[a] Data derived from published studies [8•, 16••, 28•, 32, 33, 39–47, 48••]:

[b] Overall summarizes unique samples and is lower than the sum of subgroup samples, as some samples were classified into more than one subgroup

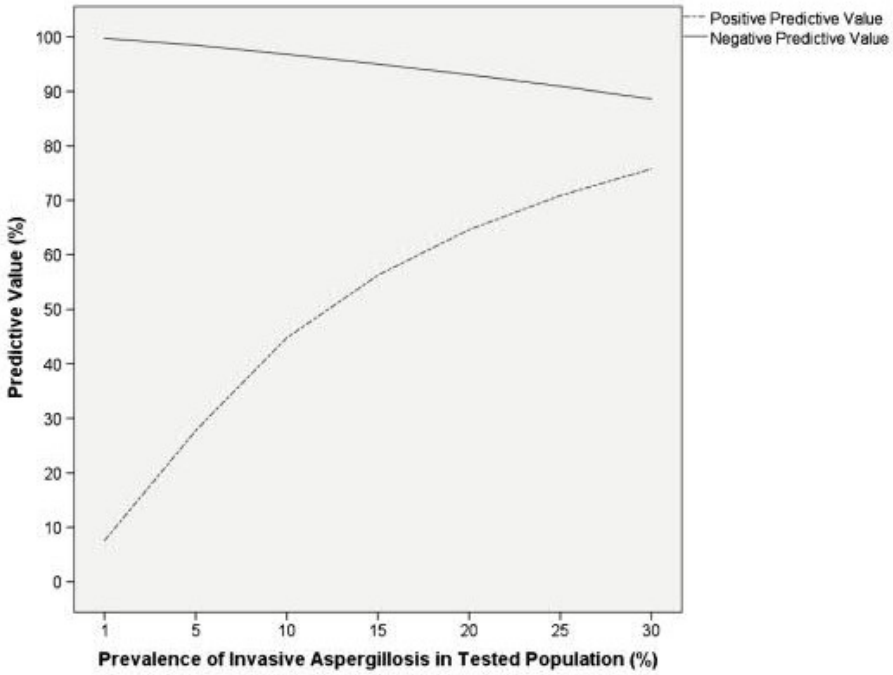

Fig. 1 Overall positive and negative predictive values of the bronchoalveolar lavage fluid *Aspergillus*-specific lateral flow device test in cohorts with prevalence rates of invasive aspergillosis between 1 and 30%. The overall sensitivity of 73% and specificity of 90% were used for the calculation

Table 2 summarizes LFD results in 127 samples from patients with possible IPA according to modified EORTC/MSG criteria [36, 49], as well as patient subgroups. The LFD resulted positive in 39% of possible IA samples versus 10% of samples from patients with no evidence of IA. When considering that diagnostic test performance calculations in the field of IA are limited by the insensitivity of all current diagnostics (including GM), these results may indicate that the LFD has additional discriminatory power in those with possible IPA, i.e., positive LFD results may provide evidence that some of those with possible IPA do in fact have (probable) IPA and a false negative GM test result. If true, positive BALF LFD test results should be considered as a future mycological factor (in addition to GM, culture, and PCR) for updated EORTC/MSG criteria.

When comparing different patient populations (Table 1), LFD sensitivity was lower in patients with underlying hematologic malignancies (67% sensitivity) compared to other patient groups. The most likely reason for lower sensitivity in patients with hematological malignancies is the frequent use of antifungal prophylaxis/empirical antifungal therapy that is beneficiary for survival in this patient population [50–56]. Similar to other fungal diagnostics, sensitivity of the LFD was reduced in the presence of antifungal prophylaxis/treatment (sensitivity 56% in those with mold active antifungals versus 86% in those without; $p = 0.0097$ Fisher's exact test; Table 3) [8•]. The solution is combining the LFD with other biomarkers such as GM in BALF and/or serum, PCR, or novel biomarkers such as triacetylfusarinine C (TAFC) [14••,

Table 2 Performance of the BALF *Aspergillus* LFD in cases of possible invasive pulmonary aspergillosis (per BALF sample) in different patient cohorts.[a]

Patient group	Positive LFD Result Percentage (absolute numbers)	Negative LFD Result Percentage (absolute numbers)
Overall[b]	39% (50/127)	61% (77/127)
Solid organ transplantation	*33% (4/12)*	*66% (8/12)*
Intensive care unit	*37% (14/38)*	*63% (24/38)*
Respiratory diseases	*65% (20/31)*	*35% (11/31)*
Hematological malignancies	*32% (26/81)*	*68% (55/81)*

[a] Data derived from published studies [16••, 28•, 39•, 40, 41, 42••, 43••, 46, 47]

[b] Overall summarizes unique samples and is lower than the sum of subgroup samples, as some samples were classified into more than one subgroup

Table 3 Sensitivity of BALF LFD for probable/proven IPA in patients with and without antifungal prophylaxis/therapy (information only available for a proportion of cases published).[a]

	BALF LFD sensitivity for IPA overall percentage (absolute numbers)
Overall	75% (50/67)
Under mold active systemic antifungals	56% (14/25)
Without mold active antifungals	86% (36/42)

BALF bronchoalveolar lavage fluid, *IPA* invasive pulmonary aspergillosis, *LFD* lateral flow device

[a] Data derived from [8•], updated with [16••, 45, 46]

16••, 28, 43••, 45, 48••], which has been shown to increase sensitivity substantially and helps to overcome this limitation.

After issues emerged with the previous manufacturing partner, redevelopment work was undertaken by OLM diagnostics after it was given full control to develop and manufacture the assay on top of its original role as sales and marketing partner. Development work is well underway and OLM are expecting to start production over the coming months and launch the LFD by the end of 2017.

Lateral Flow Device for Galactomannan-Like Antigens in Urine

Antigen testing of urine samples may provide important advantages, including non-invasive and easy sample collection that allows for more frequent examination of large volumes, which may increase test sensitivity and also has great potential for home testing [57, 58••]. Recent studies have indicated that GM testing may be promising in urine samples [57, 59, 60], while results for urine BDG testing were less convincing [61]. Fisher and colleagues reported lower specificity of GM testing of urine specimens compared to serum (80% versus 95%) in pediatric hematologic malignancy patients; however, urine GM testing successfully identified the only case of probable IA [60]. These preliminary results were confirmed in a study conducted in adult hematologic malignancy patients by Duettmann and colleagues [62]. In that study, 242 same-day serum and urine samples were included from 75 adult patients prospectively and consecutively. Out of these 75, 10 patients met criteria for probable IA; 3 additional patients were tested positive for serum GM levels. Urine samples were not pretreated before GM testing, and urine GM levels showed a significant positive correlation with serum GM levels. Sensitivity of urine GM testing was limited and only improved when using an extremely low GM cut-off of 0.1 optical density index (ODI). With that cut-off, urine GM testing exhibited 71% sensitivity and 88% specificity for probable IA. Recently, Reischies and colleagues showed in another prospective study in adult patients with hematological malignancies that test performance in urine samples can be improved by calculation of the urine GM/creatinine ratio, which takes urine dilution into account and may be a promising diagnostic tool for patients with hematological malignancies [57]. With a threshold of 0.26 ((urine GM [ODI] × 100)/(urine creatinine [mg/dL])), the positive predictive value of 13% was low, but the negative predictive value of >98% would qualify this diagnostic method for ruling out IA in high-risk patients [57].

Given this promising results, development of a point of care (POC) test for diagnosis of IA in urine samples has the potential of impacting patient care and associated costs significantly, as such a test may allow for home testing for IA. Dufresne and others recently reported that their new monoclonal antibody MAb476 was capable to detect GM-like antigen (Ag) in urine samples [58••]. Using in vitro and animal experiments, Dufresne and others [58••] investigated renal clearance of serum GM-like Ag in mouse models infected with *A. fumigatus* and generated MAb476. MAb476-based sandwich-ELISAs (sELISA) reliably detected GM-like Ag in bronchoalveolar lavage fluid, serum, and lung tissue of neutropenic mice and urine samples from guinea pigs after infection with air-borne IA [58••]. Experiments showed a specific affinity of MAb476 to *Aspergillus* spp. (excluding *A. terreus*) as well as *Fusarium* spp., *Paecilomyces* spp., and *Trichophyton rubrum* [58••]. MAb476 therefore differed from the EBA2 antibody used in Platelia® GM EIA, which showed also affinity to *A. terreus* and *Histoplasma capsulatum* C Ag [58••]. While these two antibodies may show affinity to different epitopes of the GM-like Ag, which would explain the differences, the detailed structure of the GM-like Ag has not yet been revealed [58••].

As a next step, Dufresne and colleagues successfully constructed a MAb476-based LFD prototype for urine POC testing. The functionality of MAb476 testing was confirmed with human urine samples, which were obtained from healthy volunteers and spiked with in vitro-produced *Aspergillus* antigen [58••]. However, when retrospectively testing stored samples which were collected from 11 patients who were categorized as probable/proven IA and showed positive serum GM results, sensitivity was imperfect, as only samples from 4 out of these 11 patients also had positive test results with the MAb476-sELISA and MAb476-LFD after pretreatment [58••]. Importantly, MAb476 testing appeared to be inhibited by an unknown substance or mix of substances in human urine, and this effect positively correlated to the specific mass of the urine samples [58••]. Boiling and centrifugation were not able to abandon the inhibition, and the relevant substances appeared to weigh less than 2 kDa [58••]. Urine sample concentration (5–10 fold),

followed by desalting/dialysis (7 kDa), resulted in a nearly complete diminution of the inhibition [58••].

While these results on development of the MAb476-LFD for urine samples were promising, it has to be kept in mind that the sample size was limited and the study settings were non-clinical and animal models in large parts. Additionally, the quality of the clinical samples and other information about the patients (like the presence of antifungal treatment) could not be satisfactorily evaluated [58••]. Referring to the planned use in clinical settings, the MAb476-LFD needs further optimization to fit requirements for a reliable POC device, which is currently ongoing.

Conclusion

Important advances have been made in the development of lateral flow assays for invasive aspergillosis. Most promising is the *Aspergillus*-specific lateral-flow device test, which is based on the JF5 antibody and has shown convincing performance in multiple clinical studies, in particular in BALF samples. Commercial availability is still pending, however. Recently, an LFD prototype for urine POC testing based on MAB476 has been constructed, which is currently undergoing further evaluation in studies with bigger sample sizes. Overall, the evaluation of the diagnostic performance of new assays in IA remains problematic, as the vast majority of IA cases are "probable" cases and there is no established diagnostic test in clinical routine that provides sensitivity high enough to qualify the test as a reliable gold standard.

The search for reliable antigen-based POC tests for other molds, including mucorales, continues [63]. Recently, an enzyme-linked immunospot assay has been developed, for detection of Mucorales-specific T cells in peripheral blood samples [63]. Mucorales-specific T cells polarized to the production of T helper type 2 cytokines were associated with proven invasive mucormycosis and may be detected by immunoenzymatic assays or immunocytofluorimetric assays [63]. Once validated, that assay may represent a breakthrough in diagnosis of invasive mucormycosis [63].

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Compliance with Ethical Standards

Conflict of Interest Sven Heldt declares that he has no conflict of interest.

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Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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ORIGINAL ARTICLE

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Levels of interleukin (IL)-6 and IL-8 are elevated in serum and bronchoalveolar lavage fluid of haematological patients with invasive pulmonary aspergillosis

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Summary

Aspergillus spp. have been shown to induce T-helper cell (Th) 1 and Th17 subsets resulting in elevated levels of several cytokines. The objective of this study was to analyse a bundle of cytokines in serum and bronchoalveolar lavage fluid (BALF) in patients with and without invasive pulmonary aspergillosis (IPA). This nested case-control analysis included 10 patients with probable/proven IPA and 20 matched controls without evidence of IPA, out of a pool of prospectively enrolled (2014-2017) adult cases with underlying haematological malignancies and suspected pulmonary infection. Serum samples were collected within 24 hours of BALF sampling. All samples were stored at −70°C for retrospective determination of cytokines. IL-6 and IL-8 were significantly associated with IPA in both serum ($P = .011$ and $P = .028$) and BALF ($P = .006$ and $P = .012$, respectively), and a trend was observed for serum IL-10 ($P = .059$). In multivariate conditional logistic regression analysis, IL-10 remained a significant predictor of IPA in serum and IL-8 among BALF cytokines. In conclusion, levels of IL-6 and IL-8 were significantly associated with probable/proven IPA, and a similar trend was observed for serum IL-10. Future cohort studies should determine the diagnostic potential of these cytokines for IPA, and evaluate combinations with other IPA biomarkers/diagnostic tests.

KEYWORDS
Aspergillus, BAL, haematological malignancy, IFN-γ, IL-10, IL-17A, serum

Original data of this manuscript have been presented in part at ECCMID 2017, Vienna, Austria (poster presentation number 0989) 51st Annual Meeting DMYKg 2017, Muenster, Germany (oral presentation), and TIMM 2017, Belgrade, Serbia (poster presentation).

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[3] Copy of "Levels of interleukin (IL)-6 and IL-8 are elevated in serum and bronchoalveolar lavage fluid of haematological patients with invasive pulmonary aspergillosis," by S. Heldt, S. Eigl, J. Prattes, H. Flick, J. Rabensteiner, F. Prüller, T. Niedrist, P. Neumeister, A. Wölfler, H. Strohmaier, R. Krause, and M. Hoenigl, 2017, *Mycoses*, *60*(12), pp. 818–825 (https://doi.org/10.1111/myc.12679) (81). No modifications were made. Copyright 2017 by Blackwell Verlag GmbH. Reprinted with permission.

1 | INTRODUCTION

Invasive pulmonary aspergillosis (IPA) is associated with high morbidity and mortality among patients with underlying haematological malignancies.[1,2] Due to the crude mortality of 80%-90% in the absence of adequate treatment, timely diagnosis and early start of antifungal therapy are key factors in the successful treatment of IPA.[3,4] The introduction of non-cultural diagnostic tests for IPA in blood and bronchoalveolar lavage fluid (BALF), including galactomannan antigen (GM) testing,[5,6] PCR,[7,8] the *Aspergillus*-specific lateral flow device test[9-12] and 1,3-β-D-glucan (BDG) testing,[13] was associated with a significant increase in the rate of IPA-diagnosed premortem (vs postmortem).[14] Despite these significant advancements, performance of these non-cultural diagnostic tests is varying, and the search for a reliable gold standard for diagnosis of IPA premortem continues.

Performance of currently available biomarkers may be enhanced by combination with sensitive and specific immunological markers. In fact, *Aspergillus* spp. have been shown to induce T-helper cell (Th) 1 and Th17 subsets resulting in elevated levels of several cytokines.[15,16] However, diagnostic potential of these immunological markers for diagnosis of IPA in a clinical setting has not been evaluated yet. The objective of this nested case-control analysis of a prospective cohort study was to analyse a bundle of cytokines in serum and BALF in adult patients with underlying haematological malignancies with and without IPA.

2 | MATERIALS AND METHODS

This nested case-control study of prospectively collected data comprised paired routine serum and BALF samples obtained on the same day from 10 cases with IPA and 20 matched controls without IPA.

In total, 106 patients with haematological malignancies undergoing bronchoscopy were prospectively enrolled at the Medical University of Graz, Austria, between April 2014 and March 2017. Key inclusion criteria were (i) adult patients with (ii) underlying haematological malignancy (confirmed in all but one case who was admitted to the ICU and died within hours of admission and bronchoscopy, before the haematological malignancy could be confirmed) who were (iii) at risk for IPA according to the attending clinicians (eg, febrile neutropenia, induction chemotherapy for acute myeloid leukaemia, allogeneic stem cell transplantation) and had (iv) a BALF sample obtained in clinical routine due to suspicion of infection. All patients who met inclusion criteria between April 2014 and March 2017 and signed informed consent where included in the cohort. GM was routinely performed in all serum and BALF samples and IPA was graded in accordance with the revised criteria by the European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group (EORTC) and the Mycoses Study Group of the National Institute of Allergy and Infectious Disease (MSG).[17]

A total of 10 patients had probable or proven IPA and serum plus BALF samples available (n = 8 probable IA and n = 2 proven IA) and were included in this analysis. These 10 cases were matched with each 2 controls out of a pool of 56 patients not fulfilling IPA criteria enrolled into this cohort (ie, 34 patients with possible IPA and/or missing serum/BALF sample were excluded). Controls were individually matched to cases by factors determined previously to potentially influence outcome and cytokine levels: (i) presence and duration of neutropenia,[18] (ii) presence/absence of (a) allogeneic/autologous stem cell transplantation,[19] and (b) graft vs host disease (GVHD),[19,20] (iii) underlying diseases,[18,21] (iv) receipt of corticosteroids within a week before bronchoscopy[22] and (v) viral infection within 2 weeks before bronchoscopy (ie, detection of virus in blood or BALF using nucleic acid amplification testing).[21]

Serum samples of study participants were collected at the day of bronchoscopy and stored at −70°C for retrospective cytokine measurement. Cytokine concentrations of participants enrolled into this nested case-control study were determined by the Core Facility Imaging at the Center for Medical Research of the Medical University of Graz, Austria, between September 2016 and April 2017 with a ProcartaPlex® 11plex immunoassay (eBioscience, Vienna, Austria). Investigators measuring cytokine levels were blinded towards classification of cases into IPA categories and all other clinical and demographic information. The 11 cytokines in the immunoassay were selected based on published literature in human and animal models showing an increase or decrease of these cytokines in blood and/or BALF of cases with IPA.[15,23-36] The cytokines studied were: interleukin (IL)-4, IL-6, IL-8, IL-10, IL-15, IL-17A, IL-22, soluble IL-2 receptor (sIL-2r), tumours necrosis factor (TNF) α, interferon (IFN) γ and RANTES (chemokine ligand 5). Twenty-five microlitres of undiluted freshly thawed serum samples were processed in 96-well plates according to the manufacturer's instructions using magnetic beads. Standards for each cytokine were assayed in duplicates to generate standard curves using the reference concentrations as provided by the manufacturer. Data were obtained on a validated and calibrated Bio-Plex 200 system (Bio-Rad, Vienna, Austria) and analysed with Bio-Plex Manager 6.1 software (Bio-Rad, Vienna, Austria). The cytokine concentration was calculated from the standard curve using 5PL curve fitting. Cytokine levels below the standard range were extrapolated to give approximate values. Levels of cytokines are displayed in pg/mL.

Our study was conducted in accordance with the Declaration of Helsinki, 1996, Good Clinical Practice and applicable local regulatory requirements and law. The study protocol was approved by the local ethics committees, Medical University Graz, Austria (EC-numbers 25-221 and 23-343) and registered at ClinicalTrials.Gov (Identifier: NCT02058316 and NCT01576653). Statistical analysis was performed using SPSS, version 23 (SPSS Inc., Chicago, IL, USA). Categorical data are displayed as proportions, continuous data as medians plus interquartile range (IQR) or means plus 95% confidence interval (95% CI) as appropriate. Comparisons between patient groups were performed using chi-squared test for proportions, the Mann-Whitney U test and Kruskal-Wallis test for nonparametric data. The *P* values were not corrected for multiple comparisons and are therefore only descriptive. Receiver operating characteristic (ROC) curve analyses were performed and area under the curve (AUC) values are presented including 95% CI, for cytokine levels using two approaches: (i) including extrapolated levels if below the standard range, and (ii)

levels below the standard range set to 0. Optimal cut-offs for discriminating patients with and without IPA were calculated by using the Youdens index. Utilising Cox survival analysis in SPSS, conditional logistic regression models were calculated for matched case-control pairs utilising these optimal cut-offs and adjusted for covariates used for matching, and hazard ratios (HR) including 95% CI were displayed. The sample size of 30 (with 10 cases and 20 controls) gave us 80% power (α = .05) to detect a HR of 3.0 or above. Two-sided P<.05 was taken as cut-off for statistical significance.

3 | RESULTS

A total of 30 patients were included in the final analysis. Ten patients with proven (n = 2) or probable [n = 8; all 8 had a BALF GM ≥1 optical density index and 2/8 also had a positive serum GM result (≥0.5 optical density index) on the same day] IPA, and 20 patients classified as not having IPA according to EORTC/MSG 2008 criteria. Patients' characteristics are displayed in Table 1.

For all patients corresponding concurrent BALF and serum samples were collected within a time frame of <24 hours. Median, IQR, minimum and maximum cytokine levels in cases and controls are depicted in Table 2. Box plots for IL-6, IL-8, and IL-10 in serum are depicted in Figure 1, box plots for IL-6 and IL-8 in BALF are depicted in Figure 2.

AUCs for serum cytokines for differentiating between cases and controls with and without extrapolated levels are depicted in Table 3. When including extrapolated levels IL-6, IL-8, IL-10 and IL-17A were all significantly associated with IPA with AUCs between 0.73 and 0.81. After removing extrapolated levels below the standard range, however, only IL-6 and IL-8 were significantly associated with IPA and a trend was observed for IL-10 (P = .059). AUCs for BALF cytokines are also depicted in Table 3. Both IL-6 and IL-8 were significantly associated with IPA with AUCs of 0.810 and 0.785, respectively. ROC curves for IL-6, IL-8 and IL-10 in serum are displayed in Figure 3A, ROC curves for IL-6 and IL-8 in BALF are displayed in Figure 3B.

Cut-offs calculated for serum by using Youdens index as well as results of univariate conditional logistic regression analysis for predicting the event of IPA are depicted in Table 4. In multivariate conditional logistic regression analysis of serum cytokines, only IL-10 (cut-off 6.75 pg/mL; HR 10.568, 95% CI: 1.255-89.005; P = .030) remained a significant predictor of IPA, while IL-6, IL-8 as well as covariates used for matching were not significant. In multivariate conditional logistic regression analysis of BALF cytokines only IL-8 (cut-off 710 pg/mL;

TABLE 1 Demographic data and underlying diseases of cases with probable/proven invasive pulmonary aspergillosis (IPA) and controls without evidence for IPA

Demographic data, underlying diseases and other characteristics at the time of sampling	Probable/proven IPA (n = 10)	No evidence for IPA (n = 20)
Sex		
Female	5 (50%)	14 (70%)
Male	5 (50%)	6 (30%)
Age, y		
Range	48-73	26-74
Median	54.5	60
Underlying diseases		
AML	5 (50%)	10 (50%)
NHL	1 (10%)	2 (10%)
MM	1 (10%)	2 (10%)
ALL	2 (20%)	3 (15%)
Others[a]	1 (10%)	3 (15%)
Other characteristics		
Autologous SCT	1 (10%)	2 (10%)
Allogeneic SCT	3 (30%)	7 (35%)
GvHD	2 (20%)	5 (25%)
Systemic corticosteroid treatment within 14 d of sampling	4 (40%)	4 (20%)
Viral infection with immunomodulating viruses diagnosed within 14 d of sampling	3 (30%)	5 (25%)
Neutropenia (<500/μL) ≤10 d	3 (30%)	5 (25%)
Neutropenia >10 d (<500/μL)	3 (30%)	4 (20%)

[a]Included cases of aplastic anaemia, chronic lymphatic leukaemia and active tuberculosis.
ALL, acute lymphocytic leukaemia; AML, acute myelogenous leukaemia; IPA, invasive pulmonary aspergillosis; MM, multiple myeloma; NHL, Non-Hodgkin lymphoma; SCT, stem cell transplantation; GvHD, graft vs host disease.

TABLE 2 Median and interquartile range (IQR), as well as minimum (Min) and maximum (Max) of cytokine levels (pg/mL) in IPA cases (n = 10) vs matched controls without IPA (n = 20)

		Probable/proven invasive pulmonary aspergillosis				No evidence for invasive pulmonary aspergillosis			
Material	Cytokine	Median	IQR	Min	Max	Median	IQR	Min	Max
Serum	IFNγ	2.73	1.64-5.60	1.29	39.30	2.17	1.37-3.28	0.76	4.97
	IL-10	7.66	0.41-37.46	0	358.7	0	0-1.94	0	220.2
	IL-15	1.77	0-4.48	0	14.85	0	0-0	0	9.52
	IL-17A	0.39	0-1.58	0	3.65	0	0-0.2	0	0.65
	sIL-2R	66 654	22 210-104 229	11 325	158 267	44 086	32 786-66 060	9951	113 667
	IL-22	0	0-120.4	0	813.7	0	0-0	0	140.0
	IL-4	0.12	0-4.55	0	10.83	0	0-0.17	0	12.80
	IL-6	292.5	16.98-1645	7.76	3049	9.90	3.68-36.64	0	3045
	IL-8	225.3	11.76-938.4	1.98	2359	9.37	1.64-17.78	0.41	490.5
	RANTES	65.84	34.95-451.5	21.74	608.2	34.57	23.76-89.65	0	362.9
	TNFα	2.66	1.37-3.75	0.98	4.25	1.54	1.26-2.92	0.92	3.65
BALF	IFNγ	1.69	1.29-2.09	0.82	5.43	1.65	1.29-1.84	0.94	2.22
	IL-10	0	0-1.50	0	15.96	0	0-0	0	2.98
	IL-15	4.11	2.15-4.11	0	7.33	4.11	0-11.64	0	38.20
	IL-17A	0.80	0-4.32	0	5.04	0.12	0-1.23	0	8.58
	sIL-2R	253	139.5-533.9	0	1193	125.1	48.97-356.2	0	3689
	IL-22	72.01	31.82-177.1	0	281.3	59.5	0-187.6	0	797.0
	IL-4	1.95	1.35-4.05	0.87	11.25	4.05	2.33-5.78	1.02	7.67
	IL-6	635.9	126.2-2267	56.30	2953	33.11	12.69-85.10	5.89	70 685
	IL-8	1731	940.2-3916	263.7	5706	450.7	194.3-792.8	80.57	5673
	RANTES	8.74	3.40-31.38	2.62	109.8	6.92	2.61-16.79	0.88	34.14
	TNFα	2.14	1.49-3.49	0.92	6.19	1.47	0.96-2.53	0.62	17.32

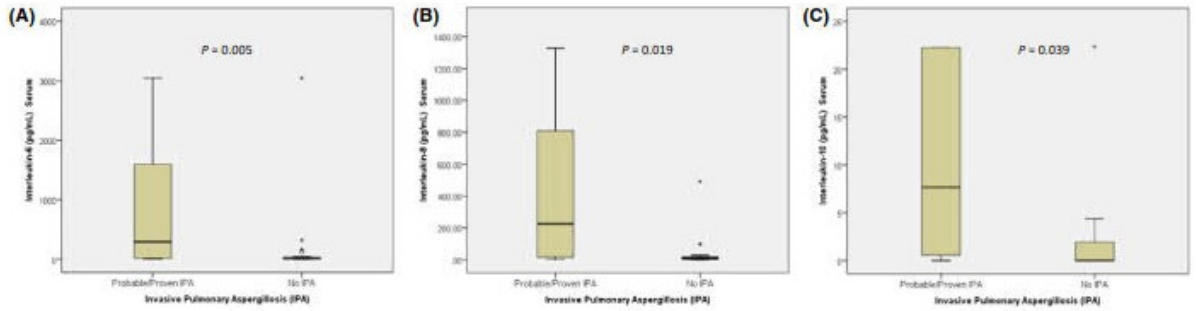

FIGURE 1 Box plots of serum interleukin (IL) 6 (A), IL-8 (B) and IL-10 (C) serum levels in patients with probable/proven IPA vs matched controls without IPA. Extrapolated values below the standard range are included. *are outliers.

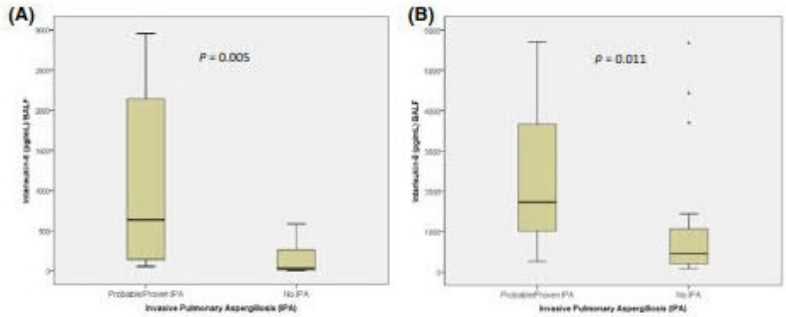

FIGURE 2 Box plots of serum interleukin (IL) 6 (A) and IL-8 (B) bronchoalveolar fluid levels in patients with probable/proven IPA vs matched controls without IPA. Extrapolated values below the standard range are included. *are outliers.

TABLE 3 Performance of cytokine levels in serum and bronchoalveolar fluid (BALF) for differentiating cases with probable/proven IPA (n = 10) from matched controls (n = 20)

	Serum (including extrapolated values if below standard range)			Serum (excluding extrapolated values if below standard range)			BALF (including extrapolated values if below Standard range)			BALF (excluding extrapolated values if below standard range)		
Cytokine	AUC	95% CI	P value	AUC	95% CI	P value	AUC	95% CI	P value	AUC	95% CI	P value
IFNγ	0.638	0.410-0.865	.226	0.550	0.322-0.778	.660	0.500	0.266-0.734	1.000	0.475	0.27256-0.694	.826
IL-10	**0.735**	**0.535-0.935**	**.039**	0.715	0.503-0.927	.059	0.570	0.347-0.793	.538	0.500	0.277-0.723	1.000
IL-15	0.690	0.474-0.906	.095	0.555	0.327-0.783	.628	0.478	0.270-0.685	.843	0.363	0.164-0.561	.226
IL-17A	**0.733**	**0.514-0.951**	**.041**	0.600	0.370-0.830	.379	0.593	0.359-0.826	.416	0.610	0.384-0.836	.333
sIL-2R	0.560	0.302-0.818	.598	0.560	0.302-0.818	.598	0.588	0.376-0.799	.441	0.588	0.376-0.799	.441
IL-22	0.605	0.380-0.830	.356	0.605	0.380-0.830	.356	0.515	0300-0.730	.895	0.515	0300-0.730	.895
IL-4	0.608	0.389-0.826	.344	0.523	0.298-0.747	.843	**0.275**	**0.066-0.485**	**.048**	0.528	0.301-0.754	.809
IL-6	**0.810**	**0.650-0.970**	**.006**	**0.790**	**0.611-0.969**	**.011**	**0.810**	**0.655-0.965**	**.006**	**0.810**	**0.655-0.965**	**.006**
IL-8	**0.765**	**0.578-0.952**	**.020**	**0.750**	**0.541-0.951**	**.028**	**0.785**	**0.617-0.953**	**.012**	**0.785**	**0.617-0.953**	**.012**
RANTES	0.650	0.438-0.862	.187	0.650	0.438-0.862	.187	0.540	0.314-0.766	.725	0.540	0.314-0.766	.725
TNFα	0.658	0.438-0.877	.166	0.500	0.277-0.723	1.000	0.655	0.453-0.857	.173	0.500	0.277-0.723	1.000

Significant differences ($P < .05$) are in bold. AUC, area under the curve; CI, confidence interval

HR: 11.685, 95% CI: 1.423-95.915; $P = .022$) remained significant, while IL-6 as well as covariates used for matching were not significant.

Among patients with probable/proven IPA, 7 out of 10 had received mould active antifungals for more than 2 days before BALF and blood samples were obtained (median: 25 days, range: 8 to >360 days). Serum levels of IL-2R ($P = .033$) and IL-22 ($P = .017$) were significantly lower in those receiving antifungals >2 days, and trends towards lower levels were also observed for IL-8, IL-10 and IL-17A. In sub-analysis of only those three patients with probable/proven IPA who had received mould active antifungals for ≤2 days before sampling and their respective matched controls, serum IL-6, IL-8, IL-22 and RANTES were significantly higher in patients with IPA, while trends were also observed for IL-10 and IL-2R. In BALF, only IL-17A levels were significantly lower in those receiving antifungals >2 days versus those IPA cases without antifungals, with similar trends observed for IL-6 and IL-2R (all analyses after removal of extrapolated levels).

4 | DISCUSSION

In this nested case-control study, we investigated the diagnostic performance of a bundle of cytokines in serum and BALF for diagnosing IPA among patients with underlying haematological malignancies. We found that serum and BALF concentrations of IL-6 and IL-8 were significantly higher in patients with probable/proven IPA compared to those without evidence of IPA. We also found a trend towards elevated serum levels of IL-10, and in multivariate conditional logistic regression analysis serum IL-10 levels ≥6.75 pg/mL remained the sole predictor of probable/proven IPA.

Our main finding was that IL-6 and IL-8 levels in serum and BALF were significantly elevated in patients with probable/proven IPA vs controls with suspected pulmonary infection but no evidence of IPA. In median IL-6 and IL-8 levels were 20-30 times higher in serum and 4-20 times higher in BALF of those with probable/proven IPA vs those without evidence of IPA. Both cytokines are centrally involved in protective immunity against *Aspergillus* spp. In early stages of IPA, conidia are killed by local alveolar macrophages, and IL-8, also known as neutrophil chemotactic factor, is produced by macrophages and epithelial cells as an important chemoattractant for neutrophils.[15] Adaptive immunity develops when dendritic cells present fungal peptides to *Aspergillus*-specific CD^{4+}-naive T cells.[15] IL-6 plays an important role in T-cell recruitment and promotes differentiation of *Aspergillus*-specific CD^{4+}-naive T cells into Th2 and Th17 cells,[15,37] thereby influencing the Th1/Th2 balance which is known to be a critical factor determining the outcome of invasive fungal infections.[38]

The mechanism of IL-6 and IL-8 increase during IPA has been studied in a number of in vitro studies. In 1999, Borger and colleagues have reported an up-regulation of gene transcription by *Aspergillus fumigatus* proteases as cause of increased release of IL-6 and IL-8 by A549 pulmonary epithelial cells and primary epithelial cells.[39] More recent studies have shown that in vitro opsonisation of *A. fumigatus* conidia with H-ficolin,[40] L-ficolin [23] and M-ficolin,[41] which play essential roles in pathogen recognition and complement activation through

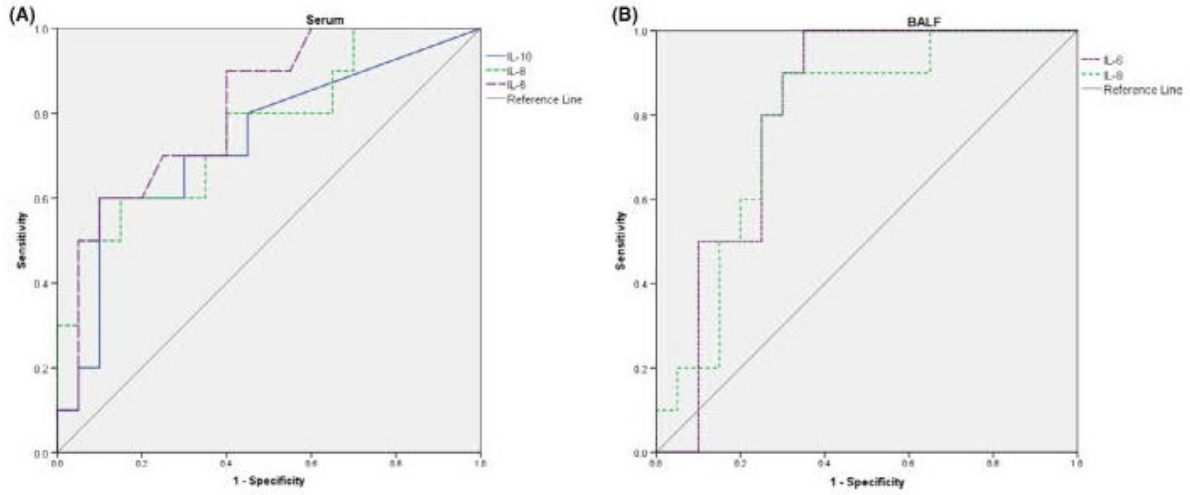

FIGURE 3 Receiver operating characteristics (ROC) curve analysis of IL-6, IL-8 and IL-10 in serum and IL-6 and IL-8 in bronchoalveolar lavage fluid (BALF) for diagnosing probable/proven invasive pulmonary aspergillosis

TABLE 4 Optimal cut-offs calculated using Youdens index for serum and BALF cytokines to differentiate cases with IPA from controls and results of conditional logistic regression analysis stratified by case-control triplets

				Conditional logistic regression (univariate)		
Cytokine	Cut-off (pg/mL)	Sensitivity	Specificity	Hazard ratios	95% confidence interval	*P* value
Serum						
IL-6	>15	90	60	8.110	−0.979-67.209	.052
	>200	60	90	10.568	1.255-89.005	.030
IL-8	>88	60	85	76.775	0.044-135 210	.255
	>360	50	95	10.000	1.168-85.594	.036
IL-10	>6.75	60	90	10.568	1.255-89.005	.030
BALF						
IL-6	>56	100	65	103.614	0.261-41 193.739	.129
IL-8	710	90	70	11.685	1.423-95.915	.022

the lectin pathway, potentiate IL-8 secretion of A549 lung epithelial cells.[42] A similar mechanism has also been proposed for infections caused by *Aspergillus flavus*.[43] After in vitro stimulation with *A. fumigatus*, Kruppel-like Factor 4 has been shown to modulate IL-6 release in human dendritic cells.[37] Dectin-1-dependent IL-6 production regulates expression of iron chelators, haem and siderophore-binding proteins and hepcidin in infected mice and reduces systemic iron levels.[16,33,44] A pivotal role for IL-6 in protective immunity against *Aspergillus* has been reported in mice.[35] While IL-6 also plays a role in the transition from innate to acquired immunity during bacterial infection,[45] IL-6 may be predominantly elevated in IPA vs other infections of the lung, including *Pneumocystis carinii* pneumonia.[46]

We also found a trend towards increased serum levels of IL-10 in patients with probable/proven IPA and in serum IL-10 was the major predictor of IPA in multivariate conditional logistic regression analysis. IL-10 is an immunosuppressive cytokine and a central negative regulator of inflammatory responses, which has been attributed a largely detrimental role during fungal disease.[16,18,29,47] In a study by Potenza and colleagues, *Aspergillus*-specific T-cells producing non-protective IL-10 and protective IFN-γ were exclusively detected in haematologic malignancy patients with invasive aspergillosis and not in uninfected controls.[30] In contrast to findings of Potenza's study, we did not find an increase of IFN-γ levels in patients with IPA. We could also not verify findings by Ceesay and colleagues who reported that baseline IL-15, IL-2R, CCL2 and MIP-1α were significantly higher, while IL-4 was lower in patients with proven/probable invasive fungal infection compared to those with no evidence of fungal infection.[34] The latter study included a variety of fungal diseases, including yeast infections with very different immunological characteristics,[48] which may explain the difference to our nested case-control study which focused exclusively on IPA. IL-17A levels found in this study were very low in patients and controls, and once we excluded levels that were extrapolated below the standard range, not significantly different between cases and controls. This is in accordance with a previous study showing that *Aspergillus* is

a poor inducer of IL-17.[49] As an important limitation our nested case-matched control study design, which generally provides a better evidence level than a classic case-control study,[50] does not allow us to draw any conclusions regarding the added benefit of cytokine testing in addition to, for example, GM testing or PCR. Future larger cohort studies are needed to determine whether the diagnostic potential of IL-6, IL-8 and IL-10, without taking into account multiple covariates that may also result in higher cytokine levels, holds value for clinical routine. Also controls included in this study had suspected pulmonary infection for which they were undergoing routine bronchoscopy and microbiological workup of BALF samples. Other pulmonary infections may have caused increased levels of certain cytokines in controls explaining findings of this study that differ from previous studies which used uninfected controls. Cytokine levels may also vary according to underlying diseases and conditions for which our conditional analysis accounted for, however, larger studies are needed to evaluate whether cytokine levels can only be interpreted when taking into account these conditions. Finally, our study, although severely underpowered for subanalysis, showed some trends towards cytokines having less discriminatory power among those with ongoing mould-active antifungals. Future larger studies are also needed to evaluate this observation.

In conclusion, levels of IL-6 and IL-8 were significantly higher in patients with probable/proven IPA compared to controls without evidence of IPA. A trend was also observed for serum IL-10 levels. Future cohort studies should determine the diagnostic potential of these cytokines for IPA, and evaluate combinations of these cytokines with other IPA biomarkers/diagnostic tests, such as GM and PCR.

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CONFLICTS OF INTEREST

J. Prattes received consulting fee from Gilead. A. Wölfler received speaker honoraria from Merck. M. Hoenigl received research grants from Gilead; served on the speakers' bureau of Gilead, Basilea and Merck. All other authors have no conflict of interest.

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Diagnosis of invasive aspergillosis in hematological malignancy patients: Performance of cytokines, *Asp* LFD, and *Aspergillus* PCR in same day blood and bronchoalveolar lavage samples☆

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SUMMARY

Background: *Aspergillus* spp. induce elevated levels of several cytokines. It remains unknown whether these cytokines hold value for clinical routine and enhance diagnostic performances of established and novel biomarkers/tests for invasive aspergillosis (IA).
Methods: This cohort study included 106 prospectively enrolled (2014–2017) adult cases with underlying hematological malignancies and suspected pulmonary infection undergoing bronchoscopy. Serum samples were collected within 24 hours of bronchoalveolar lavage fluid (BALF) sampling. Both, serum and BALF samples were used to evaluate diagnostic performances of the *Aspergillus*-specific lateral-flow device test (LFD), *Aspergillus* PCR, β-D-glucan, and cytokines that have shown significant associations with IA before.
Results: Among 106 cases, 11 had probable IA, and 32 possible IA; 80% received mold-active antifungals at the time of sampling. Diagnostic tests and biomarkers showed better performance in BALF versus blood, with the exception of serum interleukin (IL)-8 which was the most reliable blood biomarker. Combinations of serum IL-8 with either BALF LFD (sensitivity 100%, specificity 94%) or BALF PCR (sensitivity 91%, specificity 97%) showed promise for differentiating probable IA from no IA.
Conclusions: High serum IL-8 levels were highly specific, and when combined with either the BALF *Aspergillus*-specific LFD, or BALF *Aspergillus* PCR also highly sensitive for diagnosis of IA.

Introduction

Invasive mold infections (IMI) including invasive aspergillosis (IA) are associated with high morbidity and mortality among patients with underlying hematological malignancies.[1,2] Mycological diagnosis is challenging, with cultures of bronchoalveolar lavage fluid (BALF) having a low sensitivity.[3,4] Consequently, fungal biomarkers such as galactomannan [GM], beta-D-glucan [BDG] and also molecular diagnostic tests (polymerase chain reaction [PCR]) of blood or BALF, have emerged and are now widely used for diagnosing IA.[4,5] However, performance of these biomarkers and tests has been shown to be far from perfect, particularly among patients receiving mold-active prophylaxis or treatment, which has been shown to reduce sensitivity of diagnostic tests for IA.[5–10] Despite continuing advances in the diagnostic arsenal, with emerging new diagnostic tests such as the *Aspergillus*-specific lateral-flow device

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test [LFD], and new biomarkers[11] on the horizon, IA remains difficult to diagnose.

Performance of currently available biomarkers and tests for early diagnosis of IA and IMI may be enhanced by combination with sensitive and specific immunological markers. In fact, *Aspergillus* spp. have been shown to induce T-helper cell (Th) 1 and Th17 subsets resulting in elevated levels of several cytokines,[12,13] and recent studies have indicated – after adjusting for multiple covariates also associated with higher cytokine levels – that particularly Interleukin (IL)-8 and IL-6 may show promise as diagnostic markers.[14,15] It remains however unknown whether these cytokines hold value for clinical routine. Also the added benefit of cytokine testing in addition to, for example, GM testing or PCR has not been evaluated yet.

The objective of this prospective cohort study was to determine the diagnostic potential of IL-8, IL-6 and other cytokines, as well as established and emerging tests for IA and IMI in patients with underlying hematological malignancies in a setting that uses mold-active prophylaxis.

Materials and methods

This prospective cohort study comprised paired routine serum and BALF samples obtained on the same day from cases with underlying hematological malignancies who underwent routine bronchoscopy due to suspicion of pulmonary infections.

Study cohort

In total 122 cases undergoing bronchoscopy were prospectively enrolled at the Medical University of Graz, Austria, between April 2014 and July 2017. Key inclusion criteria were i.) adult patients with ii.) underlying hematological malignancy who were iii.) at risk for IPA according to the attending clinicians (e.g. febrile neutropenia, induction chemotherapy for acute myeloid leukemia, allogeneic stem cell transplantation) and had iv.) a BALF sample obtained in clinical routine due to suspicion of infection. All patients who met inclusion criteria between April 2014 and July 2017 and signed an informed consent were included in the cohort. After signing the informed consent serum and whole blood samples were collected at the day of bronchoscopy. A total of 16 cases had to be excluded due to the following reasons: 1.) same day blood samples (i.e. collected within 24-h) were not available ($n = 14$); 2.) BALF volume after routine testings' were insufficient for further diagnostic work up within the study protocol ($n = 1$); 3.) hematological malignancy was suspected but not confirmed because of mortality within days of admission ($n = 1$). After exclusion of these 16 cases, 106 patients remained in the final analysis.

Biomarker testing

Conventional culture as well as BALF and serum GM concentrations (Platelia EIA; Bio-Rad Laboratories, Vienna, Austria) were prospectively determined in clinical routine at the Medical University of Graz. Given that the vast majority of patients received mold-active antifungals at the time of bronchoscopy cut-offs of 0.5 GM optical density index (ODI) where used for serum and BALF, following previous evidence that the 0.5 ODI cutoff is preferable in patients on mold-active antifungals.[16]

BDG testing was performed in part prospectively and in part retrospectively at the Medical University of Graz, using the commercial available Fungitell® assay (Cape Cod Diagnostics, Falmouth, MA) with an adopted protocol suitable for use on a routine BCS XP® coagulation analyzer, as described previously.[17] BDG testing was only performed in serum samples, as BALF BDG testing yields very low specificity, which is explained by the fact that non-pathogenic *Candida* colonization in the lung is leading to high BDG values.[18–20] For serum BDG we used the recommended cut-off of ≥ 80 pg/mL to define positivity.

All blood and BALF samples were initially stored at 4 °C. Aliquots of 2 mL were shipped overnight to the Department of Hematology and Oncology, University Hospital of Mannheim, Heidelberg University where a nested *Aspergillus* PCR assay was performed prospectively in all study samples according to the protocol modified by Skladny et al. [21,22] PCR assays were performed according to the protocol for blood and BALF samples.[21] Extraction of DNA was processed according to the protocol of Sambrook et al.[22]. As an internal control, a 138-bp PCR fragment encoded by the human glucose-6-phosphate dehydrogenase gene was amplified in each clinical sample.

Remaining blood and BALF isolates were stored at −70 °C. Cytokine concentrations were determined in serum and BALF samples at the Center for Medical Research of the Medical University of Graz, Austria, between 09/2016 and 10/2017 with a personalized ProcartaPlex® 11plex immunoassay (eBioscience, Vienna, Austria) as described before.[14] Investigators measuring cytokine levels were blinded towards classification of cases into IA and IMI categories and all other clinical and demographic information. The 11 cytokines in the immunoassay were selected based on published literature in human and animal models showing an increase or decrease of these cytokines in blood and/or BALF of cases with IPA.[14,15,23–28] The cytokines measured were: IL-4, IL-6, IL-8, IL-10, IL-15, IL-17A, IL-22, soluble IL-2 receptor (sIL-2r), tumors necrosis factor (TNF) α, interferon (IFN) γ, and RANTES ("regulated on activation, normal T cell expressed and secreted", synonym: chemokine ligand 5). For inclusion into the main analyses we selected only those cytokines that have shown significant associations with IA in the previously conducted nested case-control analysis matched for multiple covariates, including neutrophil status, immunosuppressant and concomitant viral and bacterial infections.[14]

Testing with the new *Asp* LFD (OLM Diagnostics, Newcastle-upon-Tyne, United Kingdom) was performed in the Microbiology Laboratory at the Medical University of Graz in 12/2017. Stored non-hemorrhagic BALF samples where thawed, vortexed, centrifuged at 14,000 G and immediately tested by applying 70 µL of BALF supernatant to the test, as described previously,[19,29] with results read 10 and 15 min later. Following the manufacturer's instructions, hemorrhagic BALF (after being vortexed and centrifuged) and serum samples were mixed 1:2 with the accompanying buffer, vortexed, heated and centrifuged at 14,000 G. 70 µL supernatant was applied to the LFD and results were read after 15 min and also after 45 min.

IA classification and Statistical analysis

IA was graded in accordance with the revised criteria by the European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group (EORTC) and the Mycoses Study Group of the National Institute of Allergy and Infectious Disease (MSG),[30] with the exclusion of BDG as a mycological criterion. IMI was graded according to the same criteria but with the inclusion of serum BDG as mycological criterion (i.e., included only in the absence of Pneumocystis or Candida infection).

Our study was conducted in accordance with the Declaration of Helsinki, 2013, Good Clinical Practice. The study protocol was approved by the local ethics committee, Medical University Graz, Austria (EC-numbers 25–221 and 23–343), and registered at ClinicalTrials.Gov (Identifier: NCT02058316 and NCT01576653). Statistical analysis was performed using SPSS, version 24 (SPSS Inc., Chicago, IL, USA). For continuous data, including cytokine levels,

Table 1
Demographic data and underlying diseases of cases with probable, possible invasive aspergillosis (IA), and no evidence for IA as well as probable invasive mold infection (IMI).

Demographic data, underlying diseases and other characteristics at the time of sampling ($n = 106$)		Probable IA ($n = 11$)	Possible IA ($n = 32$)	No evidence for IA ($n = 63$)	p-value[a]	probable IMI ($n = 18$; including probable IA)
Sex	Female	6 (55%)	13 (41%)	34 (54%)	>0.2	7 (39%)
	Male	5 (45%)	19 (59%	29 (46%)		11 (61%)
Age [years]	Median (range)	55 (49–74)	60 (26–82)	57 (27–85)	>0.2	59 (49–79)
Underlying diseases	AML/MDS	6 (50%)	22 (60%)	23 (37%)	0.06	11 (61%)
	NHL	2 (18%)	4 (13%)	18 (29%)		3 (17%)
	MM	1 (9%)	0	8 (13%)		1 (6%)
	ALL	2 (18%)	4 (13%)	5 (8%)		3 (17%)
	Others[b]	0	2 (6%)	9 (14%)		0
Other characteristics	Autologous SCT	2 (18%)	2 (6%)	7 (11%)	>0.2	3 (17%)
	Allogeneic SCT	3 (27%)	11 (34%)	13 (21%)	>0.2	6 (33%)
	GvHD	2 (18%)	7 (22%)	7 (11%)	>0.2	5 (28%)
	Systemic corticosteroid treatment within 14 days of sampling	6 (55%)	6 (19%)	18 (29%)	0.08	8 (44%)
	Positive diagnostic test for viral infections within 14 days of sampling	5 (45%)	15 (47%)	18 (29%)	0.18	11 (61%)
	Positive diagnostic test for relevant bacterial pathogens/pneumocystis or toxoplasma in BALF[c]	1 (9%)	8 (25%)	12 (19%)	>0.2	1 (9%)
	Neutropenia (< 500/ µL) ≤ 10 days	3 (27%)	6 (19%)	11 (17%)	>0.2	4 (22%)
	Neutropenia (< 500/ µL) > 10 days	5 (45%)	15 (47%)	8 (13%)	**<0.001**	7 (39%)
	Mold-active antifungal prophylaxis/treatment (within 24 h before sampling and ≥ 2 days)	10 (91%)	30 (94%)	45 (71%)	**0.02**	16 (89%)

Abbreviations: ALL = acute lymphocytic leukemia; AML = acute myelogenous leukemia; GvHD = Graft versus Host Disease; IA = invasive aspergillosis; IMI = invasive mold infection; MDS = myelodysplastic syndrome; MM = multiple myeloma; NHL = Non-Hodgkin lymphoma; SCT = stem cell transplantation.

[a] Bold indicates p-values that met statistical significance.

[b] included in cases with possible IA 1 chronic lymphoid leukemia (CLL) and 1 primary myelofibrosis; and in cases without evidence for IMI 4 CLL, 2 primary myelofibrosis, 2 Hodgkin's lymphoma, and 1 anaplastic anemia.

[c] Bacterial pathogens: 1 in probable IA, 7 in possible IA, 8 in no IA; Pneumocystis: 1 in possible IA, 3 in no IA; Toxoplasma: 1 in no IA.

receiver operating characteristic (ROC) curves analyses were performed and area under the curve (AUC) values are presented including 95% confidence intervals (95% CI) for the outcomes probable IA diagnosis, possible/probable IA diagnosis, and probable IMI diagnosis (p-values were not corrected for multiple comparisons). Optimal cut-offs for cytokines discriminating patients with probable IA vs. no IA were calculated by using the Youdens index. Utilizing these newly defined cut-offs, performance of cytokines was compared to other tests (*Aspergillus* PCR and LFD), as well as biomarkers with established cut-offs (BDG and GM), by calculating sensitivity, specificity, positive-predictive value (PPV) and negative-predictive value (NPV), and diagnostic odds ratio (DOR) (a) for probable IA versus no IA (exclusion of possible IA cases; GM as a case defining mycological criterion was not evaluated), and (b) for possible/probable/proven IA versus no IA. Two-sided p-value < 0.05 was taken as cut-off for statistical significance.

Results

Study cohort

A total of 106 cases were included in the final analysis. Eleven cases with probable IA, 32 cases with possible IA (of which 25 had also possible IMI and 7 patients had probable IMI; two had *Scedosporium* spp. growth in cultures from respiratory samples, and 5 positive serum BDG), and 63 cases classified as not having IA according to EORTC/MSG 2008 criteria. Patients' characteristics are displayed in Table 1. Overall 85/106 (80%) cases (including 10/11 of those with probable IA and 30/32 of those with possible IA) had received ≥ 2 defined daily doses of mold-active antifungal prophylaxis or treatment at the time concurrent BALF and serum samples were collected (among those with probable IA each three received empirical echinocandin or liposomal amphotericin B treatment, one intravenous voriconazole treatment, two oral voriconazole prophylaxis, and one echinocandin/posaconazole combination treatment). Among the other cases 18 received posaconazole, 17 voriconazole, 17 caspofungin, 14 liposomal amphotericin B, 7 micafungin and 2 anidulafungin at the time of sampling.

ROC curve analysis for IA

In ROC curve analysis of cytokines serum IL-8 (AUC 0.775, 95%CI 0.618–0.932; $p = 0.004$) and serum IL-6 (AUC 0.751, 95%CI 0.604–0.898; $p = 0.008$) were significantly associated with probable IA, while other cytokines (Table 2; Supplemental Tables 1a–d and 2) and also serum BDG (AUC 0.605, 95%CI 0.425–0.784; $p = 0.271$) were not. Among BALF cytokines only IL-8 (AUC 0.732, 95%CI 0.584–0.879; $p = 0.015$) was significantly associated with probable IA. Optimal cut-offs calculated by using Youdens index for serum IL-8 were ≥ 300 pg/mL, ≥ 60 pg/mL and ≥ 14 pg/mL, for serum IL-6 ≥ 40 pg/mL, and for BALF IL-8 ≥ 60 pg/mL.

Impact of bacterial and viral infections on cytokine levels

In a total of 20 cases with possible ($n = 8$) or no IA ($n = 12$) either relevant bacterial pathogens ($n = 15$), *Pneumocystis jirovecii* ($n = 4$) or *Toxoplasma gondii* ($n = 1$) were detected in BALF samples (Table 1). Serum IL-8 (median 61 pg/mL, IQR 17–785 pg/mL vs. median 9 pg/mL, IQR 3–21 pg/mL; $p = 0.018$), serum IL-6 (median 123 pg/mL, IQR 14–808 pg/mL vs. median 24 g/mL, IQR 9–93 pg/mL; $p = 0.049$) and also BALF IL-8 (median 1756 pg/mL, IQR 713–3665 pg/mL vs. median 485 pg/mL, IQR 197–1109 pg/mL; $p = 0.012$) were all significantly higher in those with probable IA when compared to those with other non-viral infections.

Table 2
Performance of cytokine levels,[a] Galactomannan (GM) and beta-D-glucan (BDG) in serum and bronchoalveolar fluid (BALF) for differentiating cases with probable IA ($n = 11$) from those without IA ($n = 63$); those with possible/probable IA ($n = 43$) and those without IA ($n = 63$), and those with probable IMI ($n = 18$) versus without IMI ($n = 63$). Significant differences ($p < 0.05$) are bold (p-values not corrected for multiple comparisions).

Sample	Biomarker or cytokine	Test performance for differentiating probable IA versus no IA				Test performance for differentiating possible/probable IA versus no IA				Test performance for differentiating probable IMI versus no IMI			
		AUC	95% CI		p-value	AUC	95% CI		p-value	AUC	95% CI		p-value
			Lower bound	Upper bound			Lower bound	Upper bound			Lower bound	Upper bound	
BALF	**BAL GM**	–	–	–	–	0.503	0.383	0.623	>0.2	**0.761**	**0.606**	**0.916**	**0.001**
	IL-4	0.457	0.305	0.610	>0.2	0.564	0.455	0.673	>0.2	0.489	0.347	0.630	>0.2
	IL-6	0.680	0.542	0.817	0.059	0.602	0.494	0.710	0.076	0.650	0.516	0.784	0.068
	IL-8	**0.732**	**0.584**	**0.879**	**0.015**	0.582	0.471	0.692	0.154	**0.692**	**0.563**	**0.821**	**0.013**
Serum	**Serum GM**	–	–	–	–	0.607	0.496	0.718	0.061	**0.699**	**0.562**	**0.836**	**0.010**
	Serum BDG	0.605	0.425	0.784	>0.2	0.514	0.401	0.626	>0.2	**0.675**	**0.531**	**0.819**	**0.024**
	IL-6	**0.751**	**0.604**	**0.898**	**0.008**	0.562	0.452	0.673	>0.2	**0.653**	**0.513**	**0.793**	**0.049**
	IL-8	**0.775**	**0.618**	**0.932**	**0.004**	**0.641**	**0.533**	**0.748**	**0.014**	**0.727**	**0.589**	**0.864**	**0.004**
	IL-10	0.659	0.465	0.853	0.093	**0.615**	**0.505**	**0.725**	**0.045**	0.619	0.465	0.7774	0.124
	IL-17A	0.683	0.495	0.870	0.055	0.603	0.493	0.714	0.072	0.632	0.483	0.781	0.088

Abbreviations: AUC: Area under the curve; BALF = bronchoalveolar lavage fluid; BDG = beta-D-glucan; CI: Confidence interval; GM = Galactomannan; IA = invasive aspergillosis; IMI = invasive mold infection; IFN = interferon; IL = interleukin; sIL-2R = soluble IL-2 receptor; RANTES = regulated on activation, normal T cell expressed and secreted (= CCL5); TNF = tumor necrosis factor.

[a] Only cytokines that have shown significant associations with IA in the previously conducted nested case-control analysis matched for multiple covariates, including neutrophil status, immunosuppressant and concomitant viral and bacterial infections [14], were included in the primary analyses of this study.

Table 3
Performance of diagnostic tests in serum samples (IL-8, IL-6, LFD, *Aspergillus* PCR, and BDG) and in same-day BALF samples (IL-8, LFD, *Aspergillus* PCR), as well as combinations, for differentiating probable invasive aspergillosis (IA; $n = 11$) versus no evidence for IA ($n = 63$) ordered by Diagnostic Odds Ratios (DOR). In the right columns: sensitivities for possible IA/possible IMI and possible IA/probable IMI.

Test/test combination	Sensitivity	Specificity	PPV	NPV	DOR	Positivity in Possible IA/possible IMI cases	Positivity in possible IA/probable IMI cases
Serum IL-8 (> 300 pg/mL)	45% (5/11)	98% (62/63)	83% (5/6)	91% (62/68)	51.7 (5.1–518)	8% (2/25)	0% (0/7)
Serum IL-8 (> 60 pg/mL)	55% (6/11)	92% (58/63)	55% (6/11)	92% (58/63)	13.9 (3.1–62.2)	16% (4/25)	43% (3/7)
Serum IL-8 (> 14 pg/mL)	82% (9/11)	63% (40/63)	28% (9/32)	95% (40/42)	7.8 (1.6–39.4)	48% (12/25)	57% (4/7)
Serum IL-6 (> 40 pg/mL)	73% (8/11)	70% (44/63)	30% (8/27)	94% (44/47)	6.2 (1.5–25.9)	20% (5/25)	29% (2/7)
Serum LFD (45 min)	9% (1/11)	97% (61/63)	33% (1/3)	86% (61/71)	3.0 (0.3–36.9)	16% (4/25)	0
Serum BDG (> 80 pg/mL)	45% (5/11)	75% (47/63)	24% (5/21)	89% (47/53)	2.4 (0.7–9.1)	4% (1/25)[a]	86% (6/7)
Blood Aspergillus PCR	0% (0/10)	100% (55/55)	–	85% (55/65)	–	0	0
Serum LFD (15 min)	0% (0/10)	98% (54/55)	–	84% (54/64)	–	4% (1/25)	0
BALF LFD (10 min)	73% (8/11)	95% (60/63)	73% (8/11)	95% (60/63)	53.3 (9.2–310)	0	0
BALF Aspergillus PCR	27% (3/11)	98% (58/59)	75% (3/4)	91% (58/64)	21.8 (2.0–235)	4% (1/24)	0
BALF LFD (15 min)	73% (8/11)	87% (55/63)	50% (8/16)	95% (55/58)	18.3 (4.0–83.8)	12% (3/25)	43% (3/7)
BALF IL-8 (> 556 pg/mL)	91% (10/11)	48% (30/63)	23% (10/43)	97% (30/31)	9.1 (1.1–75.3)	52% (13/25)	71% (5/7)
BALF IL-8 (> 1000 pg/mL)	73% (8/11)	67% (42/63)	28% (8/29)	93% (42/45)	5.3 (1.3–22.2)	36% (9/25)	43% (3/7)
Serum IL-8 (> 300 pg/mL) AND/OR Serum IL-6 (> 40 pg/mL)	73% (8/11)	70% (44/63)	30% (8/27)	94% (44/47)	6.2 (1.5–25.9)	20% (5/25)	29% (2/7)
Serum IL-8 (> 300 pg/mL) AND/OR BALF LFD (10 min)	100% (11/11)	94% (59/63)	73% (11/15)	100% (59/59)	304 (15.3–6042)	8% (2/25)	0% (0/7)
Serum IL-8 (> 300 pg/mL) AND/OR BALF LFD (15 min)	100% (11/11)	86% (54/63)	55% (11/20)	100% (54/54)	132 (7.2–2432)	20% (5/25)	43% (3/7)
Serum IL-8 (> 300 pg/mL) AND/OR BALF Aspergillus PCR	91% (10/11)	97% (57/59)	83% (10/12)	98% (57/58)	285 (23.6–3447)	12% (3/25)	0

Abbreviations: BALF = bronchoalveolar lavage fluid; BDG = beta-D-glucan; IL = Interleukin; IMI = Invasive Mold Infection; LFD = Aspergillus specific Lateral-flow-device Test; NPV = negative predictive value; PPV = positive predictive value.

[a] BDG positive in a case with probable IA and Pneumocystis infection and therefore excluded as mycological criterion for probable IMI in that case.

Performance of biomarkers for probable IA

Table 3 shows performance of tests/biomarkers/cytokines in BALF and in same-day serum samples for differentiating probable IA versus no IA. GM results were used towards classification of IA, and true GM performance was therefore non-evaluable (performance in this study: BALF GM > 0.5 ODI sensitivity 91%, specificity 92%; serum GM > 0.5 ODI sensitivity 18%, specificity 97%). Overall, tests and biomarkers showed significantly better performance in BALF compared to blood, with the exception of serum IL-8 (cut-off > 300 pg/mL: 45% sensitivity and 98% specificity) and IL-6 (cut-off > 40; 73% sensitivity and 70% specificity) which proved to be the most reliable blood biomarkers. Performance could not be enhanced by combining serum IL-8 and serum IL-6 (Table 3), but combinations of serum IL-8 (cutoff ≥ 300 pg/mL) and BALF LFD (i.e., either one or both positive) showed 100% sensitivity (specificity 94% when LFD was read after 10 min and 86% when read after 15 min), while 91% sensitivity and 97% specificity were achieved when combining serum IL-8 (cutoff ≥ 300 pg/mL) and BALF *Aspergillus* PCR.

Performances of tests/biomarkers/cytokines in cases with possible IA/possible IMI as well as cases with possible IA but probable IMI are depicted in Table 3. All cytokines but also the BALF LFD resulted positive in a proportion of cases with possible IA but probable IMI (BDG could not be evaluated as it was used as criterion for defining probable IMI).

Table 4
Performance of diagnostic tests in serum samples (IL-8, IL-6, GM, LFD, *Aspergillus* PCR, and BDG) and in same-day BALF samples (IL-8, GM, LFD, *Aspergillus* PCR), for differentiating possible/probable invasive aspergillosis (IA; $n = 43$) versus no evidence of IA ($n = 63$).

Test/test combination[a]	Sensitivity	Specificity	PPV	NPV	DOR
Serum IL-8 (> 300 pg/mL)	16% (7/43)	98% (62/63)	88% (7/8)	63% (62/98)	12.0 (1.4–102)
Serum IL-8 (> 60 pg/mL)	30% (13/43)	92% (58/63)	72% (13/18)	66% (58/88)	5.0 (1.6–15.4)
Serum LFD (45 min)	12% (5/43)	97% (61/63)	71% (5/7)	62% (61/99)	4.0 (0.7–21.7)
Serum IL-8 (> 14 pg/mL)	58% (25/43)	63% (40/63)	52% (25/48)	83% (40/58)	2.4 (1.1–5.3)
Serum LFD (15 min)	2% (1/43)	98% (62/63)	50% (1/2)	60% (62/104)	1.5 (0.1–24.3)
Serum GM (> 0.5 ODI)	5% (2/43)	97% (61/63)	50% (2/4)	60% (61/102)	1.5 (0.2–11.0)
Serum IL-6 (> 40 pg/mL)	35% (15/43)	70% (44/63)	44% (15/34)	61% (44/72)	1.2 (0.5–2.8)
Serum BDG (> 80 pg/mL)	28% (12/43)	75% (47/63)	43% (12/28)	60% (47/78)	1.1 (0.5–2.7)
Blood Aspergillus PCR	0% (0/39)	100% (55/55)	–	59% (55/94)	–
BALF Aspergillus PCR	10% (4/42)	98% (58/59)	80% (4/5)	60% (58/96)	6.1 (0.7–56.7)
BALF LFD (10 min)	19% (8/43)	95% (60/63)	73% (8/11)	63% (60/95)	4.6 (1.1–18.4)
BALF GM (> 0.5 ODI)	23% (10/43)	92% (58/63)	67% (10/15)	64% (58/91)	3.5 (1.1–11.2)
BALF LFD (15 min)	33% (14/43)	87% (55/63)	64% (14/22)	65% (55/84)	3.3 (1.2–8.8)
BALF IL-8 (> 556 pg/mL)	65% (28/43)	48% (30/63)	46% (28/61)	67% (30/45)	1.7 (0.8–3.8)
BALF IL-8 (> 1000 pg/mL)	47% (20/43)	67% (42/63)	49% (20/41)	65% (42/65)	1.7 (0.8–3.9)

Abbreviations: BALF = bronchoalveolar lavage fluid; BDG = beta-D-glucan; IL = Interleukin; LFD = Aspergillus specific Lateral-flow-device Test; NPV = negative predictive value; PPV = positive predictive value.
[a] Combinations of IL-8 > 300pg/mL (serum) and/or positive LFD (BALF read after 10 min and 15 min, respectively), as well as IL-8 > 300 pg/mL (serum) and/or positive Aspergillus PCR (BALF), were also included.

ROC curve analysis and performance of biomarkers for possible/probable IA and probable IMI

ROC curve analysis for differentiating possible and probable IA from no IA revealed that serum IL-8 (AUC 0.641, 95%CI 0.533–0.748; $p = 0.014$), and – only when extrapolated low levels were included – also serum IL-10 (AUC 0.615, 95%CI 0.505–0.725; $p = 0.045$; after extrapolated levels were excluded AUC dropped to 0.598; $p = 0.089$) were predictive for differentiating between cases with possible/probable IA versus no IA. In contrast, other serum cytokines included into the main analysis, BALF cytokines and also serum GM (AUC 0.607), serum BDG (AUC 0.514), and BALF GM (AUC 0.503) were not discriminative (Table 2). Results for differentiating cases with probable IMI ($n = 18$) versus cases without IMI ($n = 63$) are also depicted in Table 2. Results of other cytokines that were not associated with IA in the previous nested case-control analysis are depicted in Supplemental Tables 1a–d and 2).

Performance of all tests/biomarkers/cytokines for differentiating cases with clinical/radiological signs and host factors for IA (i.e., possible or probable cases) versus no evidence for IA is depicted in Table 4 (BALF cultures resulted positive in one case only, showing growth of *Scedosporium apiospermum* in a case with possible IA and probable IMI). Among all the evaluated tests, serum IL 8 (cutoff ≥ 300 pg/mL) had the highest DOR in that evaluation.

Discussion

To date, this study represents one of the most complete prospective studies in terms of the number of diagnostic tests for IA evaluated. We found that in a high risk cohort of hematological malignancy patients, where > 80% of cases had received mold-active antifungals at the time of sampling, diagnostic tests and biomarkers in BALF showed overall better performance than those in blood. There was one exception, however: high serum IL-8 levels (and to a lesser extend also serum IL-6 levels) were significantly associated with probable IA, and serum IL-8 proved to be the most reliable blood biomarker for probable IA, with better diagnostic performance than serum GM, BDG, LFD and blood *Aspergillus* PCR. A high serum IL-8 cutoff of > 300 pg/mL was highly specific, and when combined with either the BALF LFD, or the BALF *Aspergillus* PCR, also highly sensitive, delivering nearly perfect performance for differentiating probable IA from no IA. Serum IL-8 showed also potential for differentiating patients with possible/probable or proven IA from those without IA, exhibiting the highest cut-off related DOR of all evaluated tests.

Diagnosis of mold infection is challenging, in particular in patients receiving prophylaxis or early empirical treatment with mold-active antifungals,[6,8,31] and a number of studies have recommended combination of multiple biomarkers to achieve sufficient sensitivity in this setting.[4,7,8,32] Our findings clearly show the overall superior performance of biomarkers in BALF when compared to same day blood samples in those patients. The poor results of all established biomarkers in blood samples might be explained by the high percentage of prior antifungal treatment with consecutively reduced fungal burden in peripheral blood,[33] and that this study used one time blood sample testing as opposed to serial screening. As different fungal biomarkers in blood can be found at different stages of fungal diseases, single time point testing may underestimate the diagnostic performance of distinct biomarkers.[34]

Significantly better diagnostic performances were observed for serum IL-8 and also serum IL-6 when compared to established blood biomarkers. Studies have shown that cytokines are centrally involved in protective immunity against Aspergillus spp. and other molds.[14,35] In early stages of IA, conidia are killed by local alveolar macrophages, and IL-8, also known as neutrophil chemotactic factor, is produced by macrophages and epithelial cells as an important chemoattractant for neutrophils.[12,35] The mechanism of IL-8 increase during IPA has been studied in previous in-vitro studies, reporting an up-regulation of gene transcription by *Aspergillus fumigatus* proteases as cause of increased release of IL-8 (and also IL-6 which plays an important role in T cell recruitment) by A549 pulmonary epithelial cells and primary epithelial cells.[36] Other studies have shown that in-vitro opsonization of *Aspergillus fumigatus* conidia with H-ficolin, L-ficolin[23] and M-ficolin, which play essential roles in pathogen recognition and complement activation through the lectin pathway, potentiate IL-8 secretion of A549 lung epithelial cells.[15,37,38]

We found that in this cohort of high-risk hematological malignancy patients receiving mold-active antifungals at the time of sampling, IL-8 i.) was the most reliable blood biomarker for IA and IMI, ii.) showed excellent specificity when a high cut-off was used and iii.) exhibited close to perfect performance when combined with either the BALF LFD or BALF *Aspergillus* PCR. The findings of this cohort study therefore confirm those of a smaller previous nested case-control study, which used matched controls based on a number of factors that had been described to be associated

with elevated cytokine levels, and found that levels of IL-8 and IL-6 were significantly higher in serum and BALF among cases with IPA versus matched controls.[14] In line, also another very recently published study which evaluated solely BALF cytokines, found that IL-8 was the major discriminator between IPA and no IPA.[15] The cohort study design and the collection of same day serum samples allowed us to get one step further by determining the "real-life" diagnostic potential of these cytokines for IA and IMI without taking into account numerous covariates, and to also evaluate combinations of IL-8 with other IA biomarkers/diagnostic tests, such as the LFD and PCR. Really the most important finding was that all analyses confirmed the potential clinical value of IL-8 in diagnosing IA and to a lesser extend also IMI, as a single stand-alone highly specific test, or even better a highly sensitive and specific test when combined with the BALF LFD or BALF PCR.

Limitations

As an important limitation there were no proven cases and the number of cases with probable IA and also probable IMI was low, despite the study period of more than 3 years, which is the natural result of highly effective anti-mold prophylaxis strategies in place at our center. Overall fungal infections are rare in patients receiving anti-mold prophylaxis (mostly 2–3% prevalence[39,40]), and therefore multicenter studies are needed to confirm our findings in larger cohorts. To avoid bias introduced by multiple comparisons and confounding factors, we also had to rely on results of a smaller nested matched case-control analysis for identification of cytokines that were evaluated in the primary analyses of this cohort study. Case-control pairs in this nested analysis were matched for multiple covariates that may affect cytokine levels.[17]

Conclusion

In conclusion, high serum IL-8 levels were highly specific, and when combined with either the BALF LFD, or BALF *Aspergillus* PCR, also highly sensitive, delivering nearly perfect performance for differentiating probable IA from no IA. Our study indicates that serum IL-8 testing may be a valuable addition to clinical routine for diagnosing IA and IMI in high risk patients who receive mold-active antifungals.

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Conflicts of interest

J. Prattes received consulting fee from Gilead.

A. Wölfler received speaker honoraria from Merck.

D. Buchheidt is consultant to Basilea, Gilead Sciences, Merck Sharp & Dohme/Merck; received research grants from Gilead Sciences and Pfizer; serves on the speakers' bureau of Astellas, Basilea, Gilead Sciences, Merck Sharp & Dohme/Merck, and Pfizer; received travel grants from Astellas, Gliead Sciences, Merck Sharp & Dohme/Merck, and Pfizer.

M. Hoenigl received research grants from Gilead; served on the speakers' bureau of Gilead, Basilea and Merck.

All other authors no conflict.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2018.05.001.

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Using Interleukin 6 and 8 in Blood and Bronchoalveolar Lavage Fluid to Predict Survival in Hematological Malignancy Patients With Suspected Pulmonary Mold Infection

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Background: Molds and other pathogens induce elevated levels of several cytokines, including interleukin (IL)-6 and IL-8. The objective of this study was to investigate the prognostic value of IL-6 and IL-8 as well as fungal biomarkers in blood and bronchoalveolar lavage fluid (BAL) for overall survival in patients with underlying hematological malignancies and suspected mold infection.

Methods: This cohort study included 106 prospectively enrolled adult cases undergoing bronchoscopy. Blood samples were collected within 24 h of BAL sampling and, in a subset of 62 patients, serial blood samples were collected up until 4 days after bronchoscopy. IL-6, IL-8, and other cytokines as well as galactomannan (GM) and β-D-glucan (BDG) were assayed in blood and BAL fluid and associations with overall mortality were assessed at the end of the study using receiver operating characteristic (ROC) curve analysis.

Results: Both blood IL-8 (AUC 0.731) and blood IL-6 (AUC 0.699) as well as BAL IL-6 (AUC 0.763) and BAL IL-8 (AUC 0.700) levels at the time of bronchoscopy were predictors of 30-day all-cause mortality. Increasing blood IL-6 levels between bronchoscopy and day four after bronchoscopy were significantly associated with higher 90-day mortality, with similar findings for increasing IL-8 levels. In ROC analysis the difference of blood IL-8 levels between 4 days after bronchoscopy and the day of bronchoscopy had an AUC of 0.829 (95%CI 0.71–0.95; $p < 0.001$) for predicting 90-day mortality.

 [5]

[5] Copy of "Using Interleukin 6 and 8 in Blood and Bronchoalveolar Lavage Fluid to Predict Survival in Hematological Malignancy Patients With Suspected Pulmonary Mold Infection," by S.A. Rawlings, S. Heldt, J. Prattes, S. Eigl, J.D. Jenks, H. Flick, J. Rabensteiner, F. Prüller, A. Wölfler, P. Neumeister, H. Strohmaier, R. Krause, and M. Hoenigl, 2019, *Frontiers in Immunology*, *10*, article 1798, pp. 1–9 (https://doi.org/10.3389/fimmu.2019.01798)(301). No modifications were made.

Conclusions: Elevated levels of IL-6 and IL-8 in blood or BAL fluid at the time of bronchoscopy, and rising levels in blood 4 days following bronchoscopy were predictive of mortality in these patients with underlying hematological malignancy who underwent bronchoscopy for suspected mold infection.

Keywords: hematologic malignancy, invasive mold infection, interleukin-6, interleukin-8, prognosis

INTRODUCTION

Patients with hematologic malignancies are immunocompromised with increased rates of hospital admissions and, in particular, admissions to the intensive care unit (ICU) (1). Factors that increase the risk of admission to the ICU include pneumonia caused by invasive mold infections (IMI) or other pathogens (2). IMI, including invasive aspergillosis (IA), are associated with high morbidity and mortality among patients with underlying hematological malignancies (3–7). Prognosticating survival in patients at risk for IMI remains difficult (8), although prompt identification of those most at risk for severe complications and death and early initiation of antifungal or other anti-infective therapy could lead to better outcomes (9).

Aspergillus spp. have been shown to induce T-helper cell subsets resulting in elevated levels of several cytokines (10, 11) and recent studies have indicated—after adjusting for multiple covariates also associated with higher cytokine levels—that particularly Interleukin (IL)-8 and IL-6 may show promise as diagnostic markers (12, 13). Our own work suggests that elevated levels of IL-8 in patients presenting with suspected pulmonary infection have excellent specificity (>90%) for detecting IMI (14), however whether these cytokines may also predict overall mortality and whether serial measurement of this cytokines may increase their prognostic potential remains unknown.

The objective of this analysis was to determine the potential of variations in IL-6 and IL-8, as well as established fungal biomarkers, to predict overall mortality in patients with underlying hematological malignancies and suspicion of pulmonary mold infection in a setting that uses mold-active prophylaxis.

MATERIALS AND METHODS

This prospective cohort study comprised paired routine serum and BAL samples obtained on the same day from cases with underlying hematological malignancies who underwent routine bronchoscopy due to suspicion of pulmonary mold infections. The decision was based on suspicious or non-specific radiological findings in chest computed tomography, with or without clinical laboratory findings including fungal biomarker levels. Investigators had no influence on clinical interventions (e.g., bronchoscopy) and treatment of the enrolled patients. The diagnostic potential of several biomarkers and cytokines measured in same day BAL and blood samples obtained as part of this cohort study for diagnosing IMI has been previously published (13, 14). However, the present analysis focuses on the overall prognostic potential of several cytokines and biomarkers in clinical outcomes of IMI. Approximately halfway through the study, the protocol was modified to include permission to use routinely-collected surplus plasma samples stored in the hospital laboratory for 4 days following collection. These longitudinal samples have not previously been published.

IA and IMI were graded in accordance with the revised criteria by the European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group (EORTC) and the Mycoses Study Group of the National Institute of Allergy and Infectious Disease (MSG) (15).

Study Cohort

Participants undergoing bronchoscopy were prospectively enrolled at the Medical University of Graz, Graz, Austria, between April 2014 and July 2017. Key inclusion criteria were (i) adult patients with (ii) underlying hematological malignancy who were (iii) at risk for IMI according to the attending clinicians discretion (e.g., febrile neutropenia, induction chemotherapy for acute myeloid leukemia, allogeneic stem cell transplantation), had (iv) a BAL sample obtained due to clinical suspicion of pulmonary infection, and (v) an order of fungal biomarkers from BAL [i.e., galactomannan (GM) and in a subset also panfungal or Mucorales specific polymerase chain reaction]. All patients who met inclusion criteria between April 2014 and July 2017 and signed an informed consent were included in the cohort. After informed consent was obtained, serum and whole blood samples were collected within 24 h of bronchoscopy. In the last 62 patients enrolled in the study, serial daily plasma samples were also obtained from blood samples drawn as part of routine clinical care from 4 days before bronchoscopy until 4 days after bronchoscopy. Because Investigators had no influence on blood sample drawn besides the same-day samples, sample size varied between 2 and 9 plasma samples per case. These were stored at 4°C for up to 4 days before processing for storage at −70°C and analysis.

Biomarker Testing

Conventional culture as well as BAL and serum GM concentrations (Platelia enzyme immunoassay; Bio-Rad Laboratories, Vienna, Austria) were prospectively determined in clinical routine at the Medical University of Graz. Given that the vast majority of patients received mold-active antifungals at the time of bronchoscopy, cut-offs of 0.5 GM optical density index (ODI) where used for serum and BAL, following previous

evidence that the 0.5 ODI cutoff is preferable in patients on mold-active antifungals (16).

β-D-glucan (BDG) testing was performed in part prospectively and in part retrospectively at the Medical University of Graz, using the commercially available Fungitell® assay (Cape Cod Diagnostics, Falmouth, MA, USA) with an adopted protocol suitable for use on a routine BCS XP® coagulation analyzer, as described previously (17). BDG testing was only performed in serum samples, as BAL BDG testing yields very low specificity due to non-pathogenic *Candida* colonization in the lungs and high BDG values (18–20). For serum BDG we used the recommended cut-off of ≥ 80 pg/mL to define positivity.

All blood (i.e., serum and plasma) and BAL isolates used in this study were frozen to −70°C after processing and stored for batched analysis. IL-6 and IL-8 concentrations were determined in serum, plasma, and BAL samples at the Center for Medical Research of the Medical University of Graz, Austria, between 09/2016 and 10/2017 with a personalized ProcartaPlex® immunoassay (eBioscience, Vienna, Austria) as previously described (13).

Investigators measuring biomarkers and cytokine levels were blinded toward clinical and demographic information of the patients.

Assessing Mortality

All patients enrolled in the study were followed clinically on initial admission and their medical records were reviewed after discharge. Autopsies were not routinely performed or requested as part of the study and therefore the absolute rate of autopsies on patients in the study was very low—likely reflecting the low cultural predilection for autopsy in the study country.

Our study was conducted in accordance with the Declaration of Helsinki, 2013, Good Clinical Practice. The study protocol was approved by the local ethics committee, Medical University Graz, Austria (EC-numbers 25-221 and 23-343), and registered at ClinicalTrials.gov (Identifier: NCT02058316 and NCT01576653). Informed consent was obtained from all study participants. Statistical analysis was performed using SPSS, version 25 (SPSS Inc., Chicago, IL, USA). For continuous data, including cytokine levels, receiver operating characteristic (ROC) curves analyses were performed and area under the curve (AUC) values are presented including 95% confidence intervals (95% CI) for the 30-, 90-, and 180-day overall mortality outcomes (p-values were not corrected for multiple comparisons). Optimal cut-offs for cytokines discriminating in patients who died within 30 days vs. those who survived were calculated using the Youden's index. Two-sided p-value < 0.05 was taken as cut-off for statistical significance.

RESULTS

Study Cohort

In total, 122 participants undergoing bronchoscopy were prospectively enrolled between April 2014 and July 2017. A total of 16 cases had to be excluded due to the following reasons: (i) same day blood samples (i.e., collected within 24-h) were not available ($n = 13$); (ii) BAL volume after routine testing was insufficient for further diagnostic work up within the study protocol ($n = 2$); (iii) hematological malignancy was suspected but not confirmed because of mortality within days of admission ($n = 1$). After exclusion of these 16 cases, 106 patients remained in the final analysis. Patients' characteristics are displayed in **Table 1**. Mortality was 16% (17/106) at 30 days, 27.4% (29/106) at 90 days, and 42.5% (45/106) at 180 days after study enrollment and bronchoscopy. In those with mold infections, 30-day mortality was 36% for probable/proven IA, 16% for possible IA and 13% for those without evidence for IA (for all probable/possible/no IMI 33, 12, and 13%, respectively).

Overall patients who died within 30 days were more frequently neutropenic at the time of bronchoscopy, had more frequently received T-cell suppressants, had more frequently probable or proven IMI and had received more frequently mold active prophylaxis or treatment at the time of bronchoscopy (**Table 1**). Of the 17 cases who died within 30 days of bronchoscopy (median 14 days after bronchoscopy, range 1–30 days), autopsy was performed in four cases, revealing progression of acute myeloid leukemia as cause of death in two cases, while cause of death was infectious (organized viral pneumonia, multi organ failure) in the other two cases. Of the other 13 cases who did not undergo autopsy, five had probable IMI, two possible IMI, two viral pneumonias, and each one bacterial pneumonia or systemic bacterial infection. Neutropenia and receipt of mold-active antifungals were also more frequent in those who died within 90 days, in fact every single patient who died within 90 days had received a mold-active antifungal at the time of bronchoscopy while this was 74% of patients who survived to day 90 ($p = 0.002$).

Prognostic Potential of Blood and BAL Cytokines at the Time of Bronchoscopy

In ROC curve analysis, serum IL-8 was a significant predictor of 30-day overall mortality, followed by serum IL-6, while serum GM and serum BDG were not significant predictors (**Table 2**). In BAL IL-6 and IL-8 were significant predictors of overall 30-day mortality, while GM was not (**Table 2**). Both serum IL-8 and serum IL-6 as well as BAL IL-6 were also significant predictors of 90- and 180-day cumulative overall mortality, although AUCs were lower when compared to 30-day mortality, and significance driven mostly by the predictive potential for 30-day mortality (**Table 2**). When focusing only on participants who died within 30- and 90-days, or between 90- and 180-days, AUCs were highest for serum IL-8 (AUC 0.578 and 0.592, respectively), followed by serum IL-6 (AUC 0.564 for both; all $p > 0.2$). Boxplots of serum and BAL IL-8 and IL-6 levels in those who died and those who survived are displayed in **Figure 1**. For prediction of 30-day mortality, optimal cut-offs were serum IL-8 > 13.93 pg/mL (82.4% sensitivity, 61.8% specificity), serum IL-6 > 165 pg/mL (52.9% sensitivity, 87.6% specificity), BAL IL-8 > 1,111 pg/mL (64.7% sensitivity, 69.7% specificity), and BAL IL-6 > 43.95 pg/mL (100% sensitivity, 46.1% specificity).

Sub-analyses for 30-day mortality found that (i) among those with possible, probable or proven IMI serum IL-8 (AUC

TABLE 1 | Demographic data, underlying diseases, and infections in cases who died within 30 and 90 days after bronchoscopy vs. those who survived.

Demographic data, underlying diseases and other characteristics at the time of sampling ($n = 106$)		Mortality at day 30 ($n = 17$)	Survival at day 30 ($n = 89$)	*p*-value*	Mortality at day 90 ($n = 29$)	Survival at day 90 ($n = 77$)	*p*-value*
Sex	Female	6 (35%)	47 (53%)	>0.2	10 (34%)	43 (56%)	0.050
	Male	11 (65%)	42 (47%)		19 (66%)	34 (44%)	
Age [years]	Median (range)	55 (33–66)	59 (26–85)	0.13	56 (27–79)	59 (26–85)	>0.2
Underlying diseases	AML/MDS	8 (47%)	43 (48%)	>0.2	18 (62%)	33 (43%)	>0.2
	NHL	2 (12%)	22 (25%)		4 (14%)	20 (26%)	
	MM	2 (12%)	7 (8%)		2 (7%)	7 (9%)	
	ALL	2 (12%)	9 (10%)		2 (7%)	9 (12%)	
	Others#	3 (18%)	8 (9%)		3 (10%)	8 (10%)	
Other conditions	Allogeneic stem cell transplantation	5 (29%)	23 (26%)	>0.2	8 (28%)	20 (26%)	>0.2
	Autologous stem cell transplantation	1 (6%)	9 (10%)	>0.2	1 (3%)	9 (12%)	>0.2
	Graft vs. host disease	3 (18%)	13 (15%)	>0.2	5 (17%)	11 (14%)	>0.2
	Neutropenia (<500 μL) on day of BAL	12 (71%)	34 (38%)	**0.017**	18 (62%)	28 (36%)	**0.017**
	T-Cell Suppressants within 3 months of BAL	5 (29%)	9 (10%)	**0.047**	7 (24%)	7 (9%)	0.055
	Systemic corticosteroid treatment within 14 days of sampling	5 (29%)	25 (28%)	>0.2	8 (28%)	22 (29%)	>0.2
Invasive fungal infections	Probable/proven IMI	6 (35%)	12 (13%)	**0.039**	6 (21%)	12 (16%)	>0.2
	Possible IMI	3 (18%)	22 (25%)	>0.2	9 (31%)	16 (21%)	>0.2
	Probable/proven invasive aspergillosis	4 (24%)	7 (8%)	0.07	4 (14%)	7 (9%)	>0.2
	Antimould prophylaxis$/treatment	17 (100%)	69 (78%)	**0.030**	29 (100%)	57 (74%)	**0.002**
Other infections	Positive diagnostic test for relevant bacterial pathogens, pneumocystis or toxoplasma in BAL	3 (18%)	18 (20%)	>0.2	5 (17%)	16 (21%)	>0.2
	Positive diagnostic test for bacterial infections in other samples (blood culture/biopsies/urine) within 14 days of sampling	5 (29%)	19 (21%)	>0.2	9 (31%)	15 (19%)	>0.2
	Positive diagnostic test for viral infections within 14 days of sampling	6 (35%)	32 (36%)	>0.2	10 (34%)	28 (36%)	>0.2

**Bold indicates p-values that met statistical significance.*

#Included 5 chronic lymphoid leukemia (CLL); 3 primary myelofibrosis; 2 Hodgkin's lymphoma, and 1 anaplastic anemia.

$Breakdown of antifungal prophylaxis: 19 received voriconazole, 18 received posaconazole prophyaxis at the time of sampling.

ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; BAL, bronchoalveolar lavage fluid; GvHD, Graft vs. host disease; IA, invasive aspergillosis; IMI, invasive mold infection; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-hodgkin lymphoma; SCT, stem cell transplantation.

0.650), BAL IL8 (AUC 0.693), and BAL IL6 (AUC 0.631) had prognostic potential, while serum-IL-6 did not; (ii) among those with bacterial infections, serum IL-8 (AUC 0.685), BAL IL-8 (AUC 0.756), and BAL IL-6 (AUC 0.685) had prognostic potential, while serum IL-6 did not; (iii) among those with viral infections, only BAL levels of IL-8 (AUC 0.734) and IL-6 (AUC 0.677) had prognostic potential, while serum levels did not; (iv) among those with neutropenia at the time of bronchoscopy, serum levels of IL-6 (AUC 0.691) and IL-8 (AUC 0.642) had some prognostic potential while BAL levels had not; in contrast, BAL IL-8 (AUC 0.920) and BAL IL-6 (AUC 0.862) had very strong prognostic potential among non-neutropenic patients; (v) among those on corticosteroids, BAL IL-6 (AUC 0.831) and BAL IL-8 (AUC 0.728) levels had stronger prognostic potential than serum levels; and (vi) among female patients serum IL-8 (AUC 0.862), serum IL-6 (AUC 0.771), BAL IL-8 (AUC 0.812), and BAL IL-6 (AUC 0.801) had all strong prognostic potential.

TABLE 2 | Performance of cytokine levels*, Galactomannan (GM) and Beta-D-glucan (BDG) in serum and bronchoalveolar fluid (BAL) for differentiating cases who died within 30, 90, and 180 days of bronchoscopy vs. those who survived.

Sample	Biomarker or cytokine	Test performance for predicting overall 30 days mortality (17/106)				Test performance for predicting overall 90 days mortality (29/106)				Test performance for predicting overall 180 days mortality (45/106)			
		AUC	95% CI		p-value	AUC	95% CI		p-value	AUC	95% CI		p-value
			Lower bound	Upper bound			Lower bound	Upper bound			Lower bound	Upper bound	
BAL	BAL GM	0.494	0.318	0.649	0.933	0.477	0.355	0.600	0.720	0.509	0.397	0.620	0.882
	IL-6	***0.763***	***0.663***	***0.963***	***0.001***	***0.697***	***0.570***	***0.805***	***0.003***	***0.620***	***0.509***	***0.731***	***0.036***
	IL-8	***0.700***	***0.574***	***0.926***	***0.009***	0.603	0.483	0.724	0.102	0.536	0.424	0.647	0.534
Serum	Serum GM	0.646	0.477	0.814	0.058	0.571	0.443	0.700	0.259	0.539	0.424	0.653	0.503
	Serum BDG	0.563	0.403	0.724	0.411	0.406	0.279	0.533	0.139	0.446	0.333	0.559	0.346
	IL-6	***0.699***	***0.553***	***0.845***	***0.010***	***0.647***	***0.528***	***0.766***	***0.020***	***0.627***	***0.518***	***0.735***	***0.027***
	IL-8	***0.731***	***0.621***	***0.840***	***0.003***	***0.659***	***0.540***	***0.769***	***0.013***	***0.646***	***0.541***	***0.751***	***0.011***
	IL-10	0.557	0.408	0.706	0.459	0.502	0.390	0.625	0.971	0.514	0.402	0.626	0.809

*Only cytokines that have shown significant associations with mortality in the previously conducted nested case-control analysis matched for multiple covariates, including neutrophil status, immunosuppressant and concomitant viral and bacterial infections (13), were included in the primary analyses of this study.
Significant differences ($p < 0.05$) are bold and italicized (p-values not corrected for multiple comparisons).
AUC, area under the curve; BAL, bronchoalveolar lavage fluid; CI, confidence interval; GM, galactomannan; BDG, beta-D-glucan; IA, invasive aspergillosis; IMI, invasive mold infection; IFN, interferon; IL, interleukin.

Kinetics of Blood IL-6 and IL-8 Before and After Bronchoscopy

In a subset of 62 participants, serial blood samples were obtained from 4 days preceding to 4 days following bronchoscopy (this particular subset had 30-day mortality of 14%, 90-day mortality of 28%, and 180-day mortality of 50%). Kinetics of serum IL-8 and IL-6 levels stratified by categories of overall mortality are displayed in **Figures 2**, **3** (23 samples tested for Day −4, 42 samples for Day −3, 46 samples for Day −2; 53 samples for Day −1; 62 samples for Day 0; 48 samples for Day +1; 52 samples for Day +2; 46 samples for Day +3; 49 samples for Day +4).

Between the day of bronchoscopy and 4 days following bronchoscopy (samples available from 49 participants on both time points), blood IL-6 levels increased in 14/49 (29%) participants, while blood IL-8 levels increased in 19/49 (39%) of participants. Increasing IL-6 levels at day four were significantly associated with higher 90-day mortality rates [7/13 (54%) died vs. 7/36 (19%) with stable or decreasing levels; $p = 0.031$], with similar findings for increasing IL-8 levels 4 days following bronchoscopy [9/18 (50%) died vs. 5/31 (16%); $p = 0.020$]. In ROC analysis, the difference of IL-8 levels between 4 days following bronchoscopy and the day of bronchoscopy had an AUC of 0.829 (95%CI 0.71–0.95; $p < 0.001$) for predicting 90-day mortality (AUC for IL-6 0.686; 95%CI 0.499–0.872; $p = 0.044$). AUC for IL-8 difference to predict 30-day mortality was 0.771 (95%CI 0.578–0.963; $p = 0.023$) and for 180-day mortality 0.680 (95%CI 0.529–0.831; $p = 0.031$); while differences in IL-6 levels were not significant predictors for 30- and 180-day mortality.

DISCUSSION

We evaluated prognostic potential of IL-6, IL-8, and several fungal biomarkers for overall mortality in a high-risk cohort of hematological malignancy patients (>80% of cases had received mold-active antifungals at the time of sampling) undergoing bronchoscopy for suspected pulmonary mold infection. Two major findings are evident. First, elevated levels of IL-6 or IL-8 in blood or BAL fluid at the time of bronchoscopy was associated with increased 30-day overall mortality. Second, increasing blood levels of IL-8 within the 4 days following bronchoscopy were highly predictive of overall 30- and 90-day mortality.

From prior studies, it is understood that cytokines are centrally involved in protective immunity against *Aspergillus* spp. and other molds (13, 21) and may therefore be used as an early biomarker for risk stratification regarding IA associated mortality (22). In the early stages of invasive aspergillosis (IA), conidia are killed by local alveolar macrophage and IL-8, also known as neutrophil chemotactic factor, is produced by these macrophages as well as neighboring epithelial cells as an important chemoattractant for neutrophils (11, 21). The mechanism of IL-8 increase during IA has also been studied *in vitro* where an up-regulation of gene transcription by *Aspergillus fumigatus* proteases was shown to cause increased release of IL-8 (as well as IL-6, which plays an important role in T cell recruitment) by A549 pulmonary epithelial cells and primary epithelial cells (16). Other studies have shown that

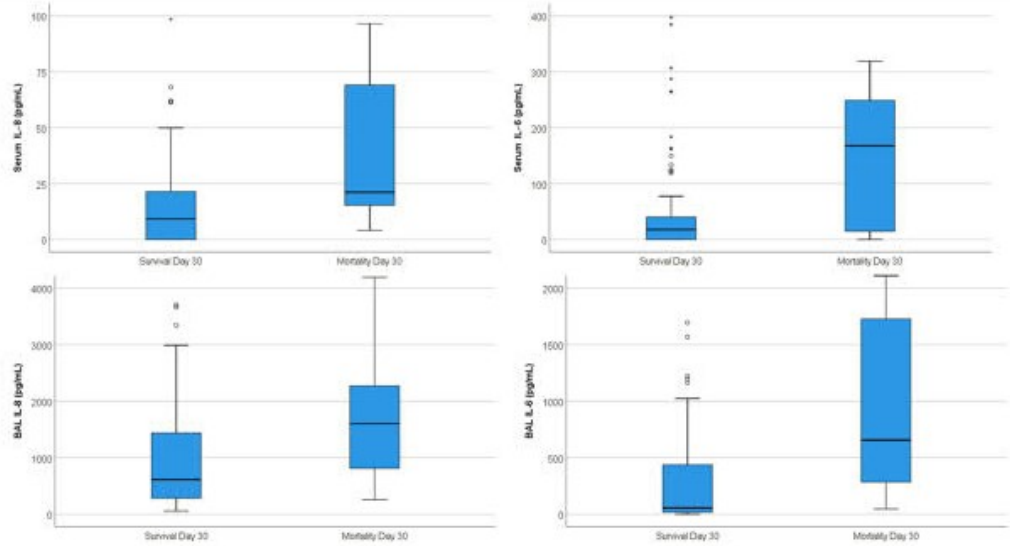

FIGURE 1 | Boxplots of same-day serum and BAL cytokines in those who survived day 30 and those who died. On the boxplot shown here, outliers are identified by different markers. "Out" values (small circle) and "far out" or as SPSS calls them "Extreme values" (marked with a star). SPSS uses a step of 1.5×IQR (Interquartile range).

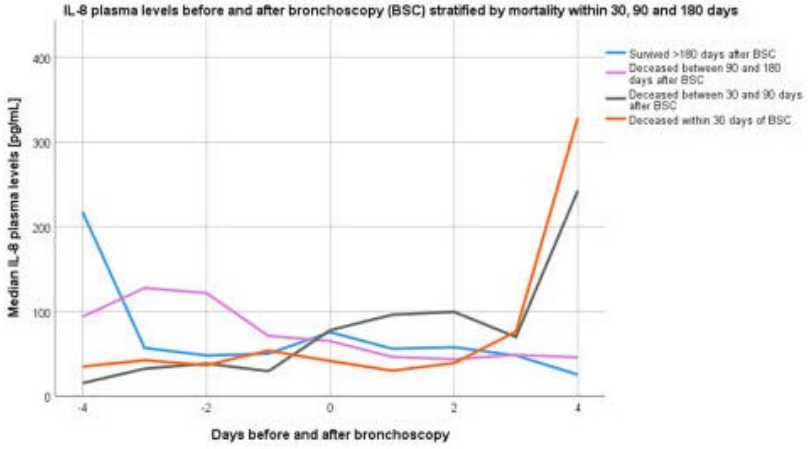

FIGURE 2 | Kinetics of median interleukin-8 plasma levels from 4 days before until 4 days after bronchoscopy stratified by overall mortality categories.

in vitro opsonization of *Aspergillus fumigatus* conidia with H-ficolin, L-ficolin (17), and M-ficolin (which play essential roles in pathogen recognition and complement activation through the lectin pathway) potentiate IL-8 secretion of A549 lung epithelial cells (12, 18, 19). In line, we have previously shown that significantly better diagnostic performances were observed for serum IL-8 and also serum IL-6 when compared to established blood biomarkers (14).

It is worth noting that a number of other conditions/irritants lead to increased levels of IL-6 and IL-8 in both blood and lung environments. Studies have shown increased levels of IL-6 and IL-8 in patients with tobacco smoke exposure and/or chronic obstructive pulmonary disease (20), asthma (23), and influenza infection (24), suggesting these are relatively non-specific cytokines involved in responses to myriad insults that may be visited upon the lungs. These other conditions, including bacterial infections, may have boosted the prognostic potential of these cytokines in our high-risk cohort where IMI was suspected but only confirmed in a subset of cases, as shown in results of our sub-analyses where cytokines were also predictive of overall mortality in those with bacterial and viral infection. As a limitation, prognostic potential of

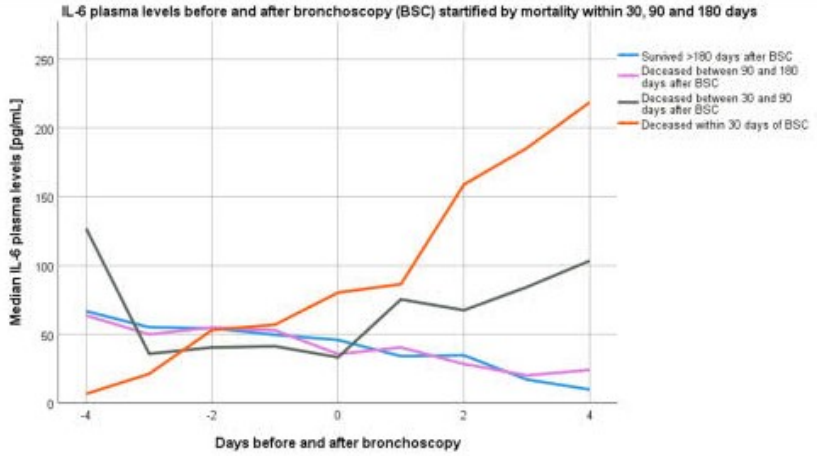

FIGURE 3 | Kinetics of median interleukin-6 plasma levels from 4 days before until 4 days after bronchoscopy stratified by overall mortality categories.

these cytokines may not be limited to patients with confirmed IMI, but may extend to patients with suspected IMI who subsequently are found to have other infections such as bacterial pneumonia. This may, however, also be considered a strength as it would allow for the use of these cytokines more broadly for treatment stratification in hematological malignancy patients with suspected pulmonary infection. While the clinical value of single measurements of cytokines may be more limited (the optimal cut-off for serum IL-8 yielded 82.4% sensitivity but only 61.8% specificity), serial measurements of these cytokines may be more promising. Overall, studies and clinical trials with larger sample sizes are needed to evaluate the prognostic potential of serial measurements of these cytokines for various subgroups of patients.

LIMITATIONS

Overall, fungal infections are rare in patients receiving anti-mold prophylaxis, with a prevalence of 2–3% (25, 26), and therefore multicenter studies are needed to confirm our findings in larger cohorts. To avoid bias introduced by multiple comparisons and confounding factors, we also had to rely on results from smaller, nested matched case-control analysis for identification of cytokines that were evaluated in the primary analyses of this cohort study. Case-control pairs in this nested analysis were matched for multiple covariates that may affect cytokine levels (27).

Additionally, autopsies were performed only in a very small subset of deceased patients and it is therefore likely that the 90- and 180-day mortality causes were not directly related to the initial reason for bronchoscopy (e.g., suspected pulmonary infection). It is still interesting that elevated levels of cytokines had predictive value for mortality so far ahead, suggesting there may be a component of specific immune dysregulation related to these cytokines playing a role in mortality in patients with underlying hematologic malignancies.

CONCLUSION

In conclusion, high blood and BAL IL-8 and IL-6 levels at the time of bronchoscopy and, in particular, increasing cytokine levels over time were predictive of mortality in a cohort of patients with underlying hematologic malignancies presenting with concern for pulmonary infection. These findings suggest it could be possible to create a treatment algorithm incorporating measurement of these cytokines at admission and throughout initial treatment for the purpose of identifying patients who warrant more aggressive treatment (e.g., combination treatment) (28, 29) when IMI is suspected in at-risk individuals.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical University Graz, Austria (EC-numbers 25-221 and 23-343). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SR, MH, JP, SE, JJ, and SH designed the study and drafted the manuscript. Data were analyzed by MH and SH. Samples were collected by SH, SE, JR, HF, AW, PN, and FP. Samples were analyzed by HS, RK, FP, JR, SH, SE, and JP. The manuscript was critically revised and important intellectual

content provided by RK, HS, HF, JR, FP, PN, and AW. The final version for publication was approved by all authors. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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