

# **Invasive fungal disease in immunocompromised hosts**

## **with focus on diagnostics**

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UNIVERSITY OF GOTHENBURG

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Cover illustration: Photomicrographs of *Pneumocystis jirovecii*, *Aspergillus fumigatus* and *Candida albicans*. Modified from Public Health Image Library, CDC. Creative Commons Lic., courtesy of Dr. Brinkman and Dr. Ewing, Jr.

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*Things are not always what they seem;  
the first appearance deceives many*

Phaedrus



# Invasive fungal disease in immunocompromised hosts

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### ABSTRACT

Invasive fungal diseases (IFDs) are severe conditions affecting immunocompromised patients. The primary aim of this thesis was to explore different methods for diagnosis of IFD in different groups of immunocompromised patients. **Papers I and II** included patients with hematologic disorders. **Paper I** was a retrospective study evaluating two years of serial 1,3- $\beta$ -d-glucan (betaglucan) testing. **Paper II** was a prospective study where samples were collected for the analysis of betaglucan, galactomannan, bm-gliotoxin (serum) and D-arabinitol/L-arabinitol (urine). The sensitivity of betaglucan and galactomannan was low early in the time course of IFD. The highest positive predictive value of betaglucan was obtained when using a cut-off level of at least 160 pg/ml and when testing patients upon clinical suspicion of IFD. Admission to ICU, previous administration of blood products and high serum triglyceride levels were associated with elevated betaglucan levels in patients without IFD. Betaglucan levels >800 pg/ml were highly indicative of IFD. Bm-gliotoxin could not be detected in patients with invasive aspergillosis. **Paper III** was a retrospective case-control study where frozen serum samples from HIV-infected patients and negative controls were analyzed for betaglucan and *Pneumocystis* PCR. *Pneumocystis* PCR in serum had a very high sensitivity and negative predictive value for the diagnosis of PCP. **Paper IV** was a prospective nationwide study on lung transplant recipients where serum and BAL-fluid samples were collected during the first post-transplant year for the analysis of betaglucan. Development of bronchiolitis obliterans syndrome (BOS) was assessed during a median 4.6 years of follow-up. Fungal colonization or tracheobronchitis had no impact on the development of BOS or on all-cause mortality. Betaglucan levels in serum were low while betaglucan levels in BAL fluid were elevated in patients with fungal tracheobronchitis. To conclude, betaglucan and *Pneumocystis* PCR in serum are useful diagnostic methods for different types of IFD although various issues need to be considered in order to determine their clinical applicability.

**Keywords:** invasive fungal disease, diagnosis, 1,3- $\beta$ -d-glucan, hematological malignancies, hematopoietic stem cell transplantation, HIV, lung transplant recipients, bronchiolitis obliterans syndrome

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# SAMMANFATTNING PÅ SVENSKA

Svampar är organismer som kan orsaka sjukdom hos människor med nedsatt immunförsvar. Invasiva svampinfektioner är allvarliga infektioner med hög dödlighet. Tidig diagnostik är avgörande för utfallet, men den är samtidigt behäftad med en rad svårigheter. Syftet med denna avhandling var att utvärdera olika metoder för diagnostik av invasiva svampinfektioner hos patientgrupper med olika typer av immunnedsättning samt att utvärdera hur vanligt det är med invasiv luftvägsinfektion orsakad av svamp och hur detta påverkar utveckling av kronisk avstötning och död hos lungtransplanterade patienter. Avhandlingen innefattar fyra arbeten. I det första arbetet utvärderades den diagnostiska nyttan av svampmarkören 1,3- $\beta$ -d-glucan (betaglucan) retrospektivt hos patienter med blodcancer och stamcellstransplanterade patienter. Betaglucan utgör en del av svampars cellvägg och kan påvisas och mätas i blodprov hos patienter med invasiv svampinfektion. Det andra arbetet utfördes prospektivt på patienter med samma bakgrundssjukdomar. Här genomgick patienterna regelbundna provtagningar för analys av svampmarkörerna betaglucan, galaktomannan och gliotoxin i blod, samt D-arabinitol/L-arabinitol i urin. Vi fann att betaglucan och galaktomannan är okänsliga som markörer för invasiv svampinfektion tidigt i infektionsförloppet men betaglucan är bättre på att diagnostisera infektionerna något senare i förloppet. Det fanns en association mellan höga betaglucan nivåer och vård på intensivvårdsavdelning, tidigare administration av intravenösa blodprodukter samt förhöjda triglycerider i blod hos patienter som inte hade invasiv svampinfektion. Betaglucannivåer  $>800$  pg/ml talade starkt för förekomsten av invasiv svampinfektion, medan bm-gliotoxin inte kunde påvisas hos någon av patienterna med invasiv svampinfektion. Det tredje arbetet syftade främst till att retrospektivt utvärdera den diagnostiska nyttan av en metod (PCR) som kan påvisa arvs massa (DNA) av svampen *Pneumocystis* i blodprov. HIV-infekterade patienter med *Pneumocystis* infektion och kontrollpatienter ingick i studien. Vi fann att 100 % av alla patienter med *Pneumocystis* infektion som ingick i studien hade påvisbart *Pneumocystis* DNA i blod vilket innebär att detta verkar vara en mycket känslig metod för diagnostik av *Pneumocystis* infektion i denna patientgrupp. Fjärde arbetet var en prospektiv undersökning på lungtransplanterade patienter med invasiv luftvägsinfektion orsakad av svamp. Vi fann att denna typ av infektion är vanlig hos lungtransplanterade patienter i Sverige, men att den inte ter sig associerad med utveckling av kronisk avstötning eller ökad dödlighet. Sammanfattningsvis, diagnostiska metoder för påvisning av betaglucan och *Pneumocystis* DNA i blod tillför nytta vid diagnostik av invasiva svampinfektioner men flera olika faktorer bör beaktas för att avgöra klinisk tillämpbarhet.

# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. **How to interpret serum levels of beta-glucan for the diagnosis of invasive fungal infections in adult high-risk hematology patients: optimal cut-off levels and confounding factors.**  
Hammarström H, Kondori N, Friman V, Wennerås C.  
*Eur J Clin Microbiol Infect Dis*. 2015;34(5):917-25
  
- II. **Prospective evaluation of a combination of fungal biomarkers for the diagnosis of invasive fungal disease in high-risk haematology patients.**  
Hammarström H, Stjärne Aspelund A, Christensson B, Heußel C.P., Isaksson J, Kondori N, Larsson L, Markowicz P, Richter J, Wennerås C, Friman V.  
*Mycoses*. 2018;61:623–632.
  
- III. **Serum-Based Diagnosis of Pneumocystis Pneumonia by Detection of Pneumocystis jirovecii DNA and 1,3-β-D-Glucan in HIV-Infected Patients.**  
Hammarström H, Grankvist A, Broman I, Kondori N, Wennerås C, Gisslen M, Friman V.  
*Submitted* 2019.
  
- IV. **Fungal colonization and tracheobronchitis following lung transplantation - impact on morbidity and mortality and utility of 1,3-β-D-glucan.**  
Hammarström H, Stjärne Aspelund A, Hansson L, Isaksson J, Kondori N, Riise GC, Wennerås C, Friman V.  
*In manuscript*.

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# ABBREVIATIONS

AA	Aplastic anemia
AIDS	Acquired immunodeficiency syndrome
ALL	Acute lymphocytic leukemia
alloHSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myeloid leukemia
BAL	Bronchoalveolar lavage
betaglukan	1,3- $\beta$ -D-glucan
Bm-gliotoxin	Bis(methyl)gliotoxin
BG	Betaglukan
BOS	Bronchiolitis obliterans syndrome
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CSF	Cerebrospinal fluid
CT	Computed tomography
Ct	Cycle threshold
DA/LA	D-arabinitol/L-arabinitol
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassay
EORTC/MSG	European Organization for Research and Treatment of Cancer /Mycoses Study Group
GC-MS	Gas chromatography mass spectrometry
GM	Galactomannan
HIV	Human immunodeficiency virus
HPLC-MS/MS	High-performance liquid chromatography tandem mass spectrometry
HR	Hazard ratio
IA	Invasive aspergillosis
ICU	Intensive care unit
IFD	Invasive fungal disease
ISHLT	International Society for Heart and Lung Transplantation
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MDS	Myelodysplastic syndrome
MRI	Magnetic resonance imaging
NPV	Negative predictive value
ODI	Optical density index
PCP	<i>Pneumocystis pneumonia</i>
PCR	Polymerase chain reaction
Pj	<i>Pneumocystis jirovecii</i>
PPV	Positive predictive value
RNA	Ribonucleic acid
ROC	Receiver operating characteristics
STARD	Standards for Reporting Diagnostic Accuracy
TOD	Time of diagnosis

# 1 INTRODUCTION

In the presence of an intact human immunological defense, the exposition to fungi seldom results in invasive infection. These so called opportunistic organisms may, however, cause severe infections in individuals with an impaired immunity. The disease process caused by the invading fungus depends on the interaction between the immunological response of the infected host and the pathogen. A variety of different diseases entities are described and the term “invasive fungal disease” (IFD) has been adopted to better reflect this perspective.

As a result of new potent immunosuppressive therapies, improved outcome after solid organ and hematopoietic stem cell transplantation, and an ageing population, the number of immunocompromised individuals has increased during the last decades. This has also led to an increased frequency of IFD. IFDs are severe conditions associated with significant morbidity and mortality, and early diagnosis and treatment is of uttermost importance for the outcome of the patients. Despite the increased frequency, IFDs are still rare conditions that typically present with uncharacteristic symptoms and a high level of suspicion is needed in order to avoid a delay in the diagnosis. However, the diagnosis of IFD is difficult and afflicted by various shortcomings, why intense research is being conducted in an attempt to improve the diagnostic procedures.

Clinical research in the field of IFD diagnostics is challenging and studies report heterogeneous results. This is partly due to the low incidence of IFD, the inherent diversity in the clinical presentation of IFD and difficulties in the case definition, but also due to issues related to the design of diagnostic studies.

This thesis will deal with IFD in different groups of immunocompromised patients. The focus will lie on diagnostic aspects of IFD and some of the difficulties in conducting diagnostic research will be highlighted.

# 1.1 The fungi

Fungi are found throughout the environment and are among the most widely distributed organisms on earth. There are 3-6 million estimated fungal species on earth and currently 120 000 accepted species<sup>1</sup>. Fungi are heterotrophic organisms, and they depend on organic carbon compounds for their nutrition. Fungi exist as saprobes (living on dead or decaying matter), symbionts (living in symbiosis with another organism), commensals (living in close relationship with another organism which neither benefits nor is harmed), or parasites (living on the expense of another organism such as a human host). Similar to animals and plants, fungi are eukaryotic organisms belonging to the Domain Eucarya (Figure 1). The majority of eukaryotic species are unicellular, while some groups such as plants, animals, and some fungi have evolved to form complex multicellular structures<sup>2</sup>.

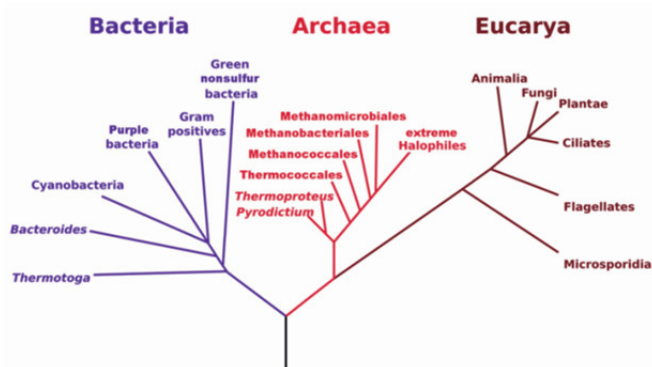


Figure 1. Phylogenetic Tree of Life. The Domain Eucarya includes the Kingdom Fungi, or Eumycota. Image Creative Commons lic. Courtesy of C. Woese.

Phylogenetic fungal taxonomy is based on the morphology and method of spore formation of the organisms<sup>3</sup>. The kingdom Fungi is subdivided in a hierarchical manner, recognized by a particular ending:

- Phylum (division): -mycota
- Subphylum (Subdivision): -mycotina
- Class: -mycetes
- Subclass: -mycetidae
- Order: -ales
- Family: -aceae
- Genus (e.g. *Candida*)
- Species (e.g. *Candida albicans*)

Despite the high estimated number of fungal species, fewer than 100 are known to be pathogenic to humans. The majority of medically important fungal species are included in the taxonomic groups, or phylum, **Zygomycota**, **Ascomycota** and **Basidiomycota**<sup>2</sup>.

In Sweden, the majority of cases of IFD are caused by fungi from the genera *Candida*, *Aspergillus* and *Pneumocystis*, but IFD caused by *Cryptococcus*, *Fusarium* and molds belonging to the order *Mucorales* are also seen<sup>4,5</sup>. Some medically important fungi are strictly confined to certain geographic regions and are usually referred to as endemic fungi. The papers included in this thesis predominantly address infections caused by *Candida*, *Aspergillus* and *Pneumocystis* and the introduction of this thesis will thus mainly focus on these genera of fungi. Endemic fungi will not be covered.

### 1.1.1 Fungal morphology and clinical classification

Medically important fungi display complex morphological features and life cycles, including both sexual and asexual reproduction mechanisms. The fungi may be unicellular or multicellular, and some species display different morphological stages depending on environmental factors. In clinical practice, human pathogenic fungi are usually classified according to their morphological features and asexual method of growth into **yeasts** and **filamentous fungi**, or **molds**<sup>2,6</sup>.

**Yeasts** are unicellular organisms that divide by budding or fission. Some medically important yeasts are *Candida* and *Cryptococcus*.

*Pneumocystis* is a **yeast-like fungus** with some characteristic morphological traits not shared by other yeasts.

**Filamentous fungi, or molds**, grow by apical extension of their filaments, forming multicellular hyphae. This hyphal growth occurs with or without cell wall separation, called septation. The hyphae may grow and form a mat-like structure called mycelium. Some medically important molds are *Aspergillus*, *Fusarium* and fungi belonging to the order *Mucorales*, such as *Rhizopus* and *Absidia*. *Aspergillus* and *Fusarium* form **septate hyphae**, while *Mucorales* are characterized by the formation of **non-septate hyphae**.

**Polymorphic fungi** are characterized by their ability to alternate between yeast-like growth and filamentous growth depending on the surrounding environment. This process is called **morphogenesis**. Polymorphic fungi may exist in yeast-form, but may also form pseudohyphae and/or true hyphae.

*Candida* has the ability to form pseudohyphae or true hyphae in addition to its yeast stage and is one of the most important polymorphic fungi.

Morphologic forms of some medically important fungi are shown below.

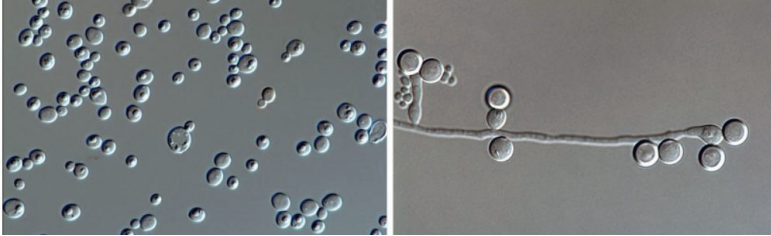


Figure 2. Budding yeast cells of *Candida albicans* (left) and the formation of pseudohyphae (right). De Hoog. *Clinical Atlas of Fungi*<sup>2</sup>. Copyright. Reprinted with permission from the Westerdijk Institute.

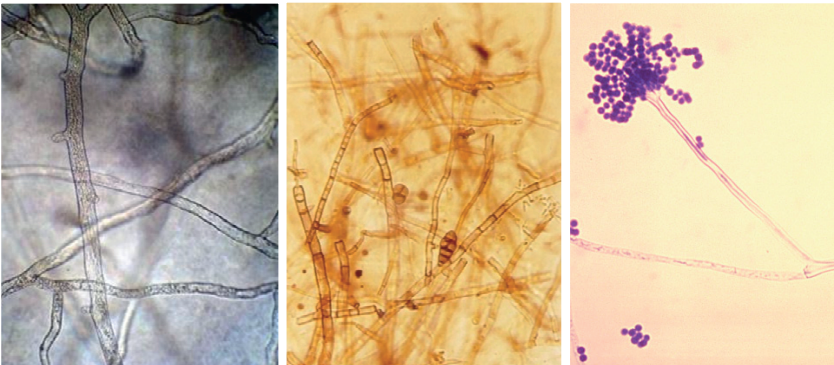


Figure 3. Non-septate hyphae from the mold *Rhizopus* (left), septate hyphae from the mold *Alternaria* (center), and conidial head with conidiospores from the mold *Aspergillus* (right). Images Creative Commons lic.



Figure 4. The different morphological stages of *P. jirovecii*. Trophic forms (A) undergo developmental stages (B) into cystic forms (ascus) containing up to eight ascospores (C). Thomas et al. *NEJM*<sup>7</sup>. Copyright. Reprinted with permission from Mass. Medical Society.

All fungal cells are coated by a **cell wall**. Although the cell wall structure varies between fungal species and between different morphological stages within the same species, the core components are mainly conserved. The cell wall is composed of an inner layer of chitin, an adjacent layer of glucans (polymers of glucose, linked mainly via  $\beta$ -(1,3) or  $\beta$ -(1,6) bonds, but in some species via  $\alpha$ -(1,3) or  $\alpha$ -(1,4) bonds), and an outer layer of glycoproteins and polysaccharides such as mannan or galactomannan<sup>8-10</sup>. Figure 5 shows the basic structure of the fungal cell wall.

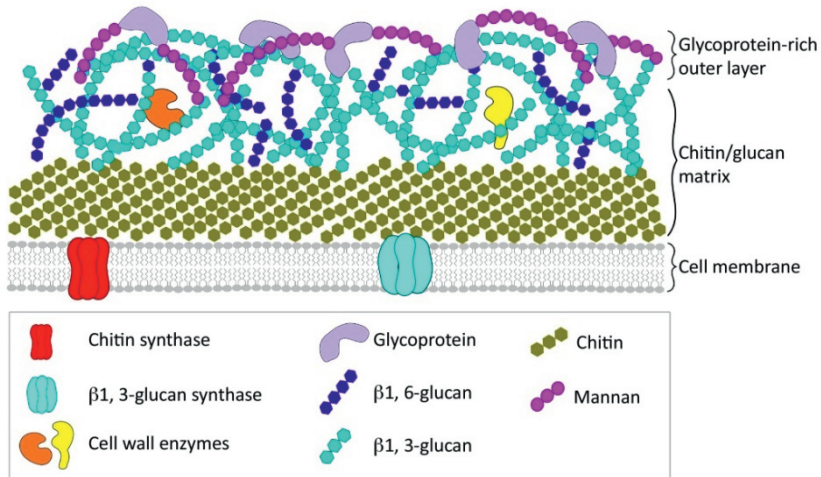


Figure 5. Basic structure of the fungal cell wall. Geoghegan et al. Trends Microbiol<sup>11</sup>. Copyright. Reprinted with permission from Elsevier.

There are some important structural differences in the cell wall components of medically important fungi which play a role in the use of diagnostic fungal antigen assays, which will be discussed later.

#### Dominating cell wall components<sup>12</sup>

<i>Candida</i>	$\beta$ -(1,3) and $\beta$ -(1,6) glucan, Chitin, Mannan
<i>Aspergillus</i>	$\beta$ -(1,3) and $\beta$ -(1,4) glucan, Chitin, Galactomannan
<i>Pneumocystis</i>	$\beta$ -(1,3) and $\beta$ -(1,6) glucan, Chitin
<i>Fusarium</i>	$\beta$ -(1,3) and $\beta$ -(1,6) glucan, Chitin
<i>Mucorales</i>	Chitin, Chitosan
<i>Cryptococcus</i>	Chitin, $\alpha$ -(1,3) glucan, Chitosan

## 1.2 Fungal-host interaction

Requirements for the development of fungal disease are the ability of the fungus to survive and grow within the infected host and the ability of the fungus to damage the host giving rise to symptoms of disease<sup>13</sup>. However, the ability to cause damage is not a property of the fungus alone, but a result of the interplay of a susceptible host and an infecting microorganism by the, so called, damage-response framework<sup>14</sup>. A wide variety of virulence factors of pathogenic fungi have been described. Some are common factors shared by all pathogenic fungi, but many virulence factors are species specific<sup>13</sup>. Although the complex interplay between fungal virulence factors and host defense mechanisms is far beyond the scope of this thesis, some aspects will be mentioned in the following sections.

### 1.2.1 Fungal virulence traits

Fungal survival and growth are mediated by different strategies of the fungus to invade tissue and to evade the immune system of the infected host<sup>13</sup>. A first pathogenic trait of medically important fungi is their ability to **survive and grow at 37 °C**<sup>15</sup>. Additionally, the **fungal cell wall** is a crucial element for the pathogenesis of fungi. The cell wall is essential both for the invasion of host tissue and for the protection of the fungal cell against the host immune response. The dynamic structure of the cell wall and the ability of fungi to alter its composition during morphological growth facilitate evasion of the host defense. Fungi display an active mode of invasion of host tissue driven by inherent fungal properties such as filamentous growth, yeast-to-hypha transition or active penetration by turgor pressure. This **morphogenesis**, facilitates tissue invasion and dissemination as well as immune response evasion and is considered an important fungal virulence factor. Furthermore, some cell wall polysaccharides are also known to function as true virulence factors<sup>8-10,13</sup>. Some additional species specific virulence traits will briefly be mentioned in section 1.4.

### 1.2.2 Host immune response

Humans are continuously exposed to fungi via the lungs, gut and skin; yet the majority of these encounters do not give rise to disease. This is mainly achieved by effective innate immune responses that recognize and eliminate the fungi<sup>9</sup>.



Non-specific mechanisms of host defense include the **first line barriers** of the exposed skin and mucous membranes of the respiratory, gastrointestinal and genitourinary tract as well as competition from the inherent microbial flora<sup>16</sup>. Invading fungal cells are then recognized by human host cells via different Pathogen Associated Molecular Patterns (PAMPs) of the fungus. This initiates a downstream cascade of events promoting the activation of the immune system. **Macrophages** in the invaded tissue, **activated by T lymphocytes**, are the first responders during fungal invasion. Early **neutrophil recruitment** to exposed tissue and subsequent neutrophil activation then results in fungal elimination by different mechanisms that are dependent upon the fungal species and the fungal morphological stage. An exception exists for *Pneumocystis* and *Cryptococcus*, where **CD4<sup>+</sup> T cells** rather than neutrophils play the important role in fungal elimination<sup>9,10,16</sup>.

In addition to inherent immune response mechanisms, recent studies have shown that human susceptibility to invasive fungal infections also may depend on specific genetic variations (single-nucleotide polymorphisms) which make certain individuals more prone to develop invasive infection<sup>17,18</sup>.

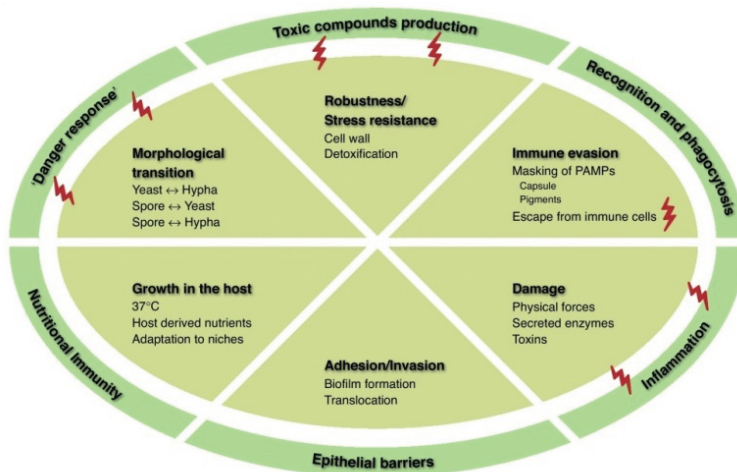


Figure 6. Schematic overview of virulence factors and human host defense mechanisms in a prototypic human pathogenic fungus<sup>13</sup>. Copyright. Reprinted with permission from Elsevier.

### 1.3 Fungal disease

Some fungi, e.g. filamentous dermatophytes, may cause superficial or cutaneous disease in fully immunocompetent individuals; however, as previously described, the majority of medically important fungi are

opportunistic microorganisms that may exist as commensal pathogens in healthy individuals but that may cause severe disease in individuals with impaired immune response mechanisms. As outlined above, the balance between pathogenicity of the fungus and the evoked immune response of the host determines the outcome after fungal exposure. Thus, the type and degree of impairment in immune response of the patient determines not only the susceptibility to different fungal species, but also the specific disease process, or clinical syndrome that is developed following fungal infection. The severity of disease is primarily influenced by the host state rather than by the pathogenicity of the fungus itself<sup>3,19</sup>.

Fungal disease is traditionally classified depending on the localization<sup>2,6</sup>:

Host defense	Disease classification	Examples
C O M P E T E N T	<u>No tissue response</u>	
	<b>Colonization</b>	<i>Candida</i> in the gut <i>Aspergillus</i> or <i>Pneumocystis</i> in the respiratory tract
	<b>Superficial infections</b>	Pityriasis versicolor Otitis externa
	<u>Various degrees of tissue response</u>	
	<b>(Muco-) cutaneous infections</b>	Tinea caused by dermatophytes Oral thrush, esophagitis caused by <i>Candida</i>
	<b>Subcutaneous infections</b>	Eumycetoma Sporotrichosis
	<u>Tissue-invasive infections</u>	
	<b>Localized</b>	<i>Aspergillus</i> tracheobronchitis <i>Aspergillus</i> necrotizing pneumonia <i>Pneumocystis</i> pneumonia
	<b>Disseminated</b>	Candidemia Angioinvasive aspergillosis Paracoccidioidomycosis
	C O M P R O M I S E D	

As shown above, tissue-invasive fungal diseases are primarily seen in immunocompromised hosts. However, some endemic fungi, e.g. *Histoplasma capsulatum* and *Coccidioides immitis* may give rise to invasive disease also in immune competent individuals although more severe disseminated forms of these mycoses primarily are seen in immunocompromised patients.

## 1.4 Some medically important fungi in Sweden

### 1.4.1 *Candida*

*Candida* spp. are opportunistic pathogens that exist as commensals colonizing the skin and mucosal surfaces of healthy individuals. Invasive disease typically arises either from translocation across the gut mucosa or via colonized central intravascular catheters.

The gut microbiota and the mucosal barrier form part of the first line of human defense against invasive *Candida* infections. An important factor of virulence include the ability of *Candida* spp. to form pseudohyphae and true hyphae as a response to micro environmental factors (morphogenesis), which facilitates translocation across mucosal barriers and tissue invasion. Furthermore, adhesion molecules on the cell wall also facilitate cell adherence and invasion of tissue. Other important traits of virulence of *Candida* spp. are their ability to form biofilm and to secrete proteinases. Monocytes, macrophages and neutrophils are critical in the early immunological defense against invading *Candida*, but also T-lymphocyte mediated cytokine responses play an important role<sup>20-22</sup>.

There are more than 20 species of *Candida* known to thrive in the human host<sup>2</sup>, however a few species account for the majority of invasive infections. *C. albicans* is the dominating pathogen, but during the last decades non-albicans species such as *C. glabrata* and *C. parapsilosis* have emerged as important pathogens although with somewhat different geographical distributions<sup>23</sup>. In Sweden, *C. albicans* accounts for 55-65% of the *Candida* spp. isolated from blood cultures<sup>5,24</sup>, with similar distribution in hematology and transplant units<sup>24</sup>. *Candida* species seem to differ in virulence. In an animal model, *C. parapsilosis* and *C. krusei* were found to be less virulent than *C. albicans*, *C. tropicalis*, and *C. glabrata*<sup>25</sup>.

A variety of patient related factors affecting the above mentioned first line human defense, and/or subsequent immunological mechanisms have been identified as risk factors for the development of invasive candidiasis<sup>23,26</sup>.

Risk factors for invasive candidiasis:

**Factors related to critical illness**

**Immunosuppression**

---

long-term ICU stay  
multiple organ failure  
abdominal surgery  
necrotizing pancreatitis  
central venous catheter  
broad spectrum antibiotics  
*Candida* colonization  
hemodialysis  
down regulation of immunological mechanisms

neutropenia  
glucocorticoid use

Immunosuppressed patients are thus at risk for invasive candidiasis not only due to their immunosuppressive disorders and/or treatments as such, but also due to factors associated with the severity of illness of the underlying immunosuppressive conditions.

Four main clinical syndromes of invasive candidiasis are described<sup>23,26,27</sup>:

- **Candidemia with no signs of organ involvement**, not seldom related to infections of **intravascular catheters**.
- **Acute disseminated candidiasis**, characterized by candidemia with concomitant metastatic organ involvement typically involving the lungs and eyes, but virtually any body site may be affected.
- **Deep-seated tissue candidiasis**, defined as *Candida* infection of a deep body site without concomitant signs of candidemia. Deep-seated infection may arise from either previous hematogenous dissemination or direct inoculation of candida species into a sterile site.
- **Chronic disseminated candidiasis**, or **hepatosplenic candidiasis**, a clinical syndrome seen almost exclusively following prolonged episodes of neutropenia in patients with hematologic malignancies. The organs that are predominantly involved are the liver and spleen, where

radiological exams typically reveal small target-like lesions. Fungal blood cultures are often negative<sup>28</sup>.

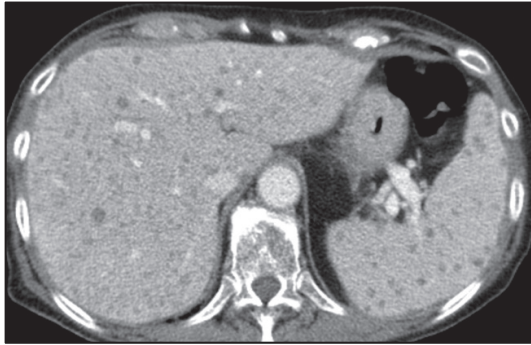


Figure 7. Portal venous phase CT with *Candida* micro abscesses in the liver and spleen in hepatosplenic candidiasis. Cornely et al. *Clinical Liver Disease*<sup>29</sup>. Copyright. Reprinted with permission from John Wiley & Sons Inc.

### 1.4.2 *Aspergillus*

*Aspergillus* spp. are ubiquitous in the environment and especially common in the soil and decaying vegetation. Humans constantly inhale numerous *Aspergillus* spores, or conidiospores, that are readily eliminated by innate immune mechanisms in immunocompetent hosts<sup>30</sup>. The first-line defenses against inhaled spores are the alveolar epithelial barrier and subsequent elimination of spores by T-cell activated alveolar macrophages. Spores that evade macrophage elimination may germinate and produce invasive hyphae. Alveolar macrophages are responsible for the initiation of a pro-inflammatory response that recruits neutrophils capable of destroying hyphae. In the absence of a functional macrophage response, spores may be allowed to germinate into hyphae within the alveolar spaces<sup>31</sup>. Depending on the degree and type of immune response impairment, the hyphae may then penetrate the respiratory epithelium and cause airway-invasive disease with a neutrophil associated inflammatory response or, in the absence of an adequate neutrophil response, subsequently penetrate the vascular endothelium giving rise to disseminated disease<sup>31</sup>. Some virulence factors that are involved in the development of tissue invasive disease include cell wall components, fungal enzymes and immunoevasive toxins, such as gliotoxin<sup>32</sup>.

Among the over 180 recognized *Aspergillus* species, *A. fumigatus* is the most common cause of human disease, also in Sweden<sup>4</sup>. Other emerging species are *A. flavus*, *A. niger*, *A. nidulans*, and *A. terreus*<sup>33</sup>.

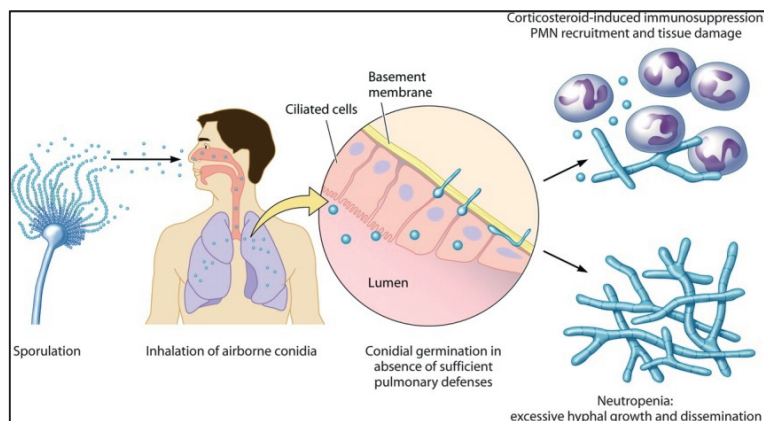


Figure 8. Image showing the establishment of *Aspergillus conidia* in different types of immunosuppressive conditions. Dagenais et al. *Clin Microbiol Rev.* 2009<sup>31</sup>. Copyright. Reprinted with permission from American Society for Microbiology.

The most important patient-related risk-factors for invasive aspergillosis are **prolonged corticosteroid therapy** and **prolonged neutropenia**<sup>31</sup>. Depending on the degree of impairment of the immunological response mechanisms described above, different clinical syndromes of invasive disease are seen<sup>30,31</sup>:

- **Invasive pulmonary aspergillosis.** The degree of neutrophil activity determines the degree of inflammatory response and the ability to limit the infection to the lung parenchyma. **Chronic necrotizing pulmonary aspergillosis** is characterized by the presence of a neutrophil response giving rise to a subacute inflammatory pneumonia with necrotic tissue lesions and cavitation. This is predominantly seen in patients with prolonged corticosteroid therapy.
- **Angio-invasive aspergillosis** may develop in the absence of a neutrophil response. Following vascular invasion, cytokines and coagulation factors are activated resulting in intravascular thrombosis, tissue ischemia and necrotic lesions<sup>34</sup>. As a result of angio-invasion, hematogenous **dissemination** may occur to other organs such as the brain and abdominal organs.

- **Tracheobronchial aspergillosis** is a separate entity of *Aspergillus* disease where the infection is limited to the tracheobronchial tissue. Tracheobronchial manifestations of *Aspergillus* may be divided according to degree of severity into tracheobronchitis, ulcerative tracheobronchitis and pseudomembranous tracheobronchitis. Tracheobronchial aspergillosis is predominantly seen in lung transplant recipients and may engage the anastomotic region of the transplanted lung<sup>35</sup>.

### **Radiology**

Early chest computer tomography (CT) scan in high-risk patients with clinical signs of infection is crucial for the diagnosis of invasive aspergillosis. Patients with pulmonary aspergillosis typically present with **characteristic radiological signs** on CT that may help in the differential diagnostics<sup>36-38</sup>:

- **Macronodules**, or well circumscribed lesions  $\geq 1$  cm (and usually  $< 3$ cm) in diameter<sup>38,39</sup>. Common finding. Low specificity.
- **Halo sign**. Macronodulus surrounded by ground-glass opacity (alveolar hemorrhage). Early sign. May be seen in angioinvasive aspergillosis during neutropenia. Higher specificity.
- **Air crescent sign. Inflammation and cavitation**. A later sign during the disease process. Is seen after recovery of neutrophils and reflects the presence of a neutrophil response.

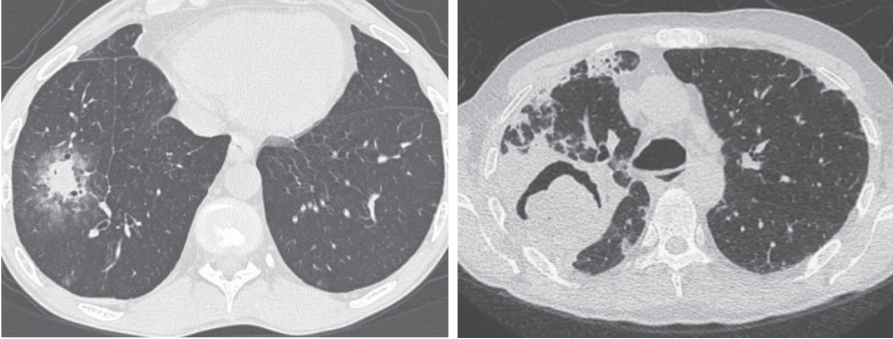


Figure 9. Thoracic CT scans of invasive aspergillosis.

(**Left**) angioinvasive aspergillosis with the characteristic 'halo sign'. Chabi et al. *Diagn Interv Imaging*<sup>40</sup>. Copyright. Reprinted with permission from Elsevier.

(**Right**) invasive pulmonary aspergillosis with the 'air crescent sign' and inflammatory necrotic lesions. Lota et al. *Thorax*<sup>41</sup>. Copyright. Reprinted with permission from BMJ Publishing Group Ltd.

### 1.4.3 *Pneumocystis*

*Pneumocystis* was originally classified as a protozoon, but was reclassified as a fungus in the 1980s. *Pneumocystis* infects only mammals; and historically, all forms of *Pneumocystis* were called *Pneumocystis carinii* followed by the host name. To date, *Pneumocystis* is known to be host species specific, and the species infecting humans has been renamed *Pneumocystis jirovecii* while *Pneumocystis carinii* is reserved for the rat form of *Pneumocystis*<sup>42</sup>. Knowledge on the basic biology of *Pneumocystis* remains limited due to the long-standing inability to reproducibly culture the organism *in vitro*. In 2014, however, Schildgen *et al* published a report stating that they successfully had cultured *P. jirovecii* in a culture system composed of an airway epithelial cell line<sup>43</sup>, yet the results have still not successfully been reproduced<sup>44,45</sup>.

*Pneumocystis* has a high tropism for the alveoli in the lungs of infected hosts where the trophic form of the fungus attaches to pneumocytes<sup>46</sup>. Knowledge about virulence factors of *Pneumocystis* is limited, but a high level of antigenic variation by selective expression of cell wall surface glycoproteins is thought to promote immune evasion and survival in the lung<sup>13</sup>. Alveolar macrophages constitute the first line of host defense against *Pneumocystis*. Among several other immunological mechanisms involved, CD4<sup>+</sup> T cells have shown to be the most critical element in the clearance of *Pneumocystis*. In contrast to the human host response against *Candida* and *Aspergillus*,



neutrophils seem to play a very limited role in the host defense against *Pneumocystis*<sup>46</sup>.

Although *Pneumocystis* is an obligate opportunistic pathogen, the fungus is believed to have an *ex vivo* spore phase that may survive in a cell-free environment<sup>46</sup>. Although insufficiently investigated, *Pneumocystis* is thought to be a ubiquitous organism, and *Pneumocystis* DNA has been detected in pond water and in air samples from both outdoor and indoor settings<sup>42</sup>. Studies have shown a high seroprevalence of *Pneumocystis* antibodies in the population, and *Pneumocystis* infection was long thought to occur as a result of reactivation of latent infection in immunosuppressed individuals. Later evidence, however, shows that *de novo* exposure either from the environment or from individuals with PCP or colonized with *Pneumocystis* may result in infection<sup>42</sup>.

*P. jirovecii* is a frequent colonizer of the respiratory tract of patients with chronic lung disease and immunosuppressive disorders such as HIV-infection, but *Pneumocystis* colonization has also been found in healthy individuals, predominantly in children<sup>47</sup>. In the presence of an impaired cellular immunological response, *Pneumocystis* may cause pneumonia.

The risk for acquiring *Pneumocystis* pneumonia (PCP) is dependent upon underlying medical conditions or the receipt of drugs that alter the T cell function, such as: **HIV-infection, lymphoproliferative disorders**, and a wide range of immunosuppressive agents such as: **glucocorticosteroids, calcineurininhibitors** (for prevention of graft versus host disease after solid organ transplantation and alloHSCT), **monoclonal antibodies, alkylating chemotherapeutic agents** and **TNF $\alpha$ -inhibitors**<sup>48</sup>.

The clinical presentation of PCP is somewhat different in patients with HIV-infection compared to patients with other immunosuppressive disorders. Typically, the following clinical pictures are seen<sup>42,49-51</sup>:

	HIV-infected patients	Non HIV-infected patients
Onset of disease	Subacute (week-months)	Acute (days)
Clinical presentation	Fever Dry cough Dyspnoea Often mild hypoxemia	Fever Dry cough Dyspnoea Often more severe hypoxemia
Burden of <i>Pneumocystis</i> organisms	High	Lower
Inflammatory response	Low	Higher
Radiological picture	Diffuse, bilateral ground-glass opacities  Cystic lesions	Bilateral, ground-glass opacities, sometimes with consolidations  Cystic lesions

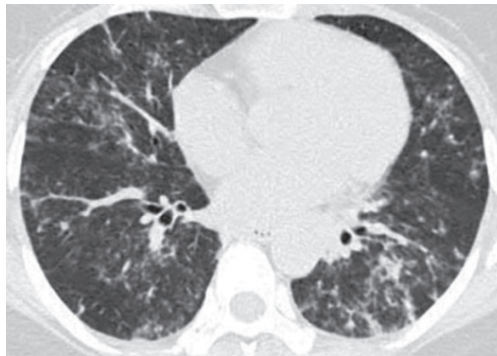


Figure 10. Typical diffuse ground-glass opacity in an HIV-infected patient with PCP. Limper et al. *Lancet Inf Dis.* 2017<sup>32</sup>. Copyright. Reprinted with permission from Elsevier. q

## 1.5 Systemic antifungals

### 1.5.1 Polyenes

The polyenes were introduced in the 1950s as the first systemic antifungals on the market. Polyenes bind to ergosterol in the fungal cell membrane leading to altered permeability and ultimately cell death. Early polyene

compounds, such as nystatin and amphotericin B deoxycholate, were, however, limited by nephrotoxic side effects.

In the 1990s, amphotericin B was reformulated with lipid-based delivery compounds resulting in compounds, e.g. **liposomal amphotericin B**, with significantly lower toxicity<sup>53</sup>. *In vitro*, amphotericin B compounds demonstrate concentration-dependent killing, and they have antifungal activity against a wide range of fungi such as *Candida* spp., *Aspergillus* spp. (with the exception of *A. terreus*), and most other filamentous fungi, such as the *Mucorales*<sup>54</sup>. Lipid formulations of amphotericin B are used for initial and salvage therapy of invasive *Aspergillus* infections<sup>55</sup> and for treatment of infections caused by other molds such as *Mucorales*<sup>56</sup>.

### 1.5.2 Flucytosine

Flucytosine is another old antifungal agent which was introduced in the 1960s. Within fungal cells, flucytosine is converted into 5-fluorouracil which acts by inhibiting fungal RNA and DNA synthesis<sup>57</sup>.

It has activity against *Candida* spp. (with the exception of *C. krusei*) and *Cryptococcus*. Monotherapy with flucytosine is limited due to a high rate of emerging resistance, but it is used in combination with amphotericin B compounds for treatment of cryptococcosis.

### 1.5.3 Azoles

Imidazoles were developed in the 1970s but have a limited use as systemic agents due to their toxicity. In the 1980s, the triazoles revolutionized medical mycology providing clinicians with systemic antifungal agents available both as intravenous and oral formulations<sup>58</sup>. Triazoles act by inhibiting the enzyme 14- $\alpha$ -lanosterol demethylase which is essential for the synthesis of ergosterol in the cell membrane of fungi. Five triazoles are currently available for use in Sweden.

**Fluconazole** is a widely used and well tolerated drug yet with a small antifungal spectrum including activity against *Candida* spp., (with the exception of some species e.g. *C. glabrata* and *C. krusei*), and *Cryptococcus*. Fluconazole is widely used as a drug for antifungal prophylaxis in settings with a low risk for mold infections, and is used as step-down therapy for invasive candidiasis caused by fluconazole-susceptible species<sup>26</sup>.

**Itraconazole** has a wide antifungal spectrum including not only activity against *Candida* spp. but also against several molds. The use of itraconazole is limited by variabilities in absorption, gastrointestinal tolerance and toxicity.

**Voriconazole** was approved in 2002 as the first of the second generation triazoles. It has good activity against *Candida* spp. and *Aspergillus* spp. and is the drug of choice for treatment of invasive aspergillosis<sup>55</sup>.

**Posaconazole** is another second generation triazole with broad antifungal spectrum including activity against *Candida* spp., *Aspergillus* spp. and some members of the *Mucorales*. Posaconazole is recommended by European guidelines as primary antifungal prophylaxis in adult patients with acute myeloid leukemia and myelodysplastic syndrome undergoing intensive remission-induction chemotherapy in settings with a high incidence of mold infections<sup>59</sup>.

**Isavuconazole**, a new extended-spectrum triazole was approved by the U.S. Food and Drug Administration in 2015. It has a broad activity against yeasts and molds, including *Mucorales* with favorable pharmacokinetic characteristics. It is approved for treatment of invasive aspergillosis and mucormycosis<sup>60</sup>.

### 1.5.4 Echinocandins

Echinocandins were introduced in the 2000s. They act by inhibiting the synthesis of 1,3- $\beta$ -D-glucan in the fungal cell wall and are the first class of antifungal agents with selective action against fungal cells without affecting mammalian cells. Echinocandins have antifungal activity against *Candida* spp (albeit with reduced *in vitro* activity against *C. parapsilosis*) and *Aspergillus* spp.<sup>61</sup>. Studies have also shown that echinocandins have activity against *Pneumocystis* spp. *in vitro* and in animal models<sup>62,63</sup>.

Three echinocandins are presently available: **caspofungin**, **micafungin** and **anidulafungin**. All echinocandins are generally well-tolerated and are recommended as the first-line treatment for candidemia<sup>26</sup>.

### 1.5.5 Others

**Trimethoprim-sulfamethoxazole** is a widely used antibacterial agent. Its mechanism of action comprises inhibition of the metabolism of folate,

consequently affecting DNA-production in microorganisms, including some fungi. Trimethoprim-sulfamethoxazole is the first-line agent for treatment of *Pneumocystis pneumonia*. Studies have also shown *in vitro* activity of sulfonamides against some *Aspergillus* spp<sup>64,65</sup>.

## 1.6 The hosts

The following chapter will focus on the epidemiology and manifestations of IFD seen in the different groups of immunocompromised patients included in **papers I-IV** of this thesis, *i.e.* patients with hematologic disorders, HIV-infected patients and lung transplant recipients.

### 1.6.1 Patients with hematologic disorders

Patients with hematologic malignancies and recipients of allogeneic hematopoietic stem cell transplantation are at risk of acquiring IFD as a result of the immunological impairment of the underlying disorder and of the immunosuppressive treatment received as well as the multiple risk factors associated with long-term hospitalization such as the presence of indwelling catheters and the use of broad-spectrum antibiotics<sup>59</sup>.

In the 1980s, **invasive candidiasis** was the predominant fungal disease in hematology units; however, along with the widespread use of azole prophylaxis since the early 1990s, invasive infections caused by *Candida albicans* have become less common<sup>66</sup>, while infections caused by non-albicans species are becoming increasingly frequent<sup>67</sup>. However, incidence numbers may differ geographically due to local routines of prophylaxis and local epidemiology<sup>5,68</sup>. Recent data on the epidemiology of candidemia in a Swedish hematology and transplant unit showed that 67% of all *Candida* isolates in blood were *C. albicans*<sup>24</sup>.

Large surveillance studies from the last decade have shown that **invasive aspergillosis** is the most common form of IFD in patients with hematologic disorders<sup>69,70</sup>, but infections caused by other molds such as *Fusarium* and molds belonging to the fungal order *Mucorales* are emerging as important fungal infections that also need to be considered in this patient cohort<sup>68,71</sup>.

The **incidence** of overall IFD in hematology units varies significantly across different settings. Studies from hematology settings in various different geographical locations, report incidence rates of IFD of 5-19% in allogeneic hematopoietic stem cell transplant (alloHSCT) recipients and patients with

hematologic malignancies<sup>69,70,72-76</sup>. These studies mainly include infections caused by molds and *Candida* spp. The **mortality** attributed to IFD in patients with hematologic disorders is high. One study reported attributable mortality rates of 39% for IFD overall, 33% for invasive candidiasis, 42% for invasive aspergillosis and 64% for mucormycosis<sup>69</sup>. Swedish data showed a similarly high mortality rate among patients with invasive mold infections in the hematology unit with an overall 90-day mortality rate of 51%<sup>4</sup>.

Before the 1980s, ***Pneumocystis pneumonia*** was recognized as an important life-threatening infection mainly in patients with acute lymphoblastic leukemia and in **HSCT recipients**. Nowadays the incidence has decreased dramatically due to the use of trimethoprim-sulfamethoxazole prophylaxis to all risk-patients, and PCP is now predominantly seen in patients that are not receiving adequate prophylaxis<sup>51</sup>.

Two important factors that recur as the predominant risk factors for contracting IFD in the hematology unit need to be highlighted:

- **Prolonged and profound neutropenia**

A factor that is crucial when determining the risk for developing IFD in the hematology unit is the duration and degree of neutropenia. Several studies report neutropenia as the major risk factor for IFD in this setting<sup>9,70,77</sup>, and patients with **acute leukemia intended for curative treatment** and **alloHSCT recipients** thus constitute major risk groups for IFD early after start of treatment for leukemia or after transplantation.

- **Systemic glucocorticoids**

Treatment with high-dose glucocorticoids significantly increases the risk for all types of IFD, where invasive candidiasis, invasive pulmonary aspergillosis and *Pneumocystis pneumonia* are among the important mycoses. One patient group that has been identified as a particularly important risk group for IFD is **alloHSCT recipients with chronic graft versus host disease** who require high doses of systemic glucocorticoids to prevent end-organ damage. In this group of patients, IFD may occur late after the period of engraftment<sup>9,21,70,78</sup>.

To conclude, in order to assess the risk for the development of IFD in patients with hematologic disorders, several factors need to be considered

such as underlying disease, dosage and duration of immune suppressive treatment, degree of neutropenia, routine for anti-fungal prophylaxis and local incidence rates<sup>59</sup>.

## 1.6.2 HIV-infected patients

Fungal infections are important contributors to the opportunistic infections seen in HIV-infected patients<sup>52</sup>. In a cohort study on AIDS-defining opportunistic illnesses in the U.S., esophageal mucosal candidiasis was the leading opportunistic illness followed by *Pneumocystis pneumonia* (incidence rates of 5 and 4 per 1000 person-years, respectively)<sup>79</sup>. **Cryptococcal meningitis** and endemic mycoses are other important invasive fungal infections in HIV-infected patients worldwide.

Before the combination antiretroviral therapy (ART) era, 70-80% of HIV-infected patients with **CD4<sup>+</sup> cell counts lower than 200 cells/μL** developed PCP with subsequently high mortality rates<sup>9</sup>. The widespread availability of antiretroviral drugs has resulted in a dramatic decrease in the incidence of HIV-related fungal opportunistic infections, yet PCP remains one of the leading AIDS-defining illnesses among patients with newly diagnosed advanced HIV-infection and patients with treatment failure or non-compliance to medication<sup>80</sup>. The mortality rate of PCP in HIV-infected patients ranges from 10% to 30% or higher in cases of severe pneumonia<sup>52,81</sup>.

## 1.6.3 Lung transplant recipients

Solid organ transplant recipients have a significant risk of contracting IFD caused mainly by *Candida* and *Aspergillus*. In a large prospective multicenter cohort study of solid organ transplant recipients, lung transplant recipients had the second highest cumulative one-year incidence of overall IFD (9%), following recipients of small bowel transplantation<sup>82</sup>.

**Invasive aspergillosis** is the most common form of IFD after lung transplantation<sup>82-84</sup> with reported incidence rates of 3-14%<sup>85</sup>. Immunosuppressive therapy consisting of an intense corticosteroid regime combined with a continuous exposure of the transplanted organ to *Aspergillus* spores, a decreased mucociliary clearance and a weakened cough reflex due surgical denervation contribute to the vulnerability to invasive aspergillosis in this patient group<sup>9,86</sup>. The clinical syndromes of *Aspergillus* disease seen in lung transplant recipients are invasive pulmonary aspergillosis and tracheobronchial aspergillosis<sup>85,87</sup>. In two multicenter

observational studies, the reported 90 days overall mortality rate in lung transplant recipients with invasive aspergillosis and invasive mold infections was 4%<sup>88</sup> and 17%<sup>89</sup>, respectively, which was lower than that of other solid organ transplant recipients.

**Invasive candidiasis** represent the second most common form of IFD after lung transplantation and is predominantly seen in the **early post-transplant period** in the form of nosocomial candidemia<sup>82,90</sup>. **Aspergillus infections**, on the other hand, typically occur **later**, within 6 months after transplantation<sup>82,91</sup>. **Candida tracheobronchitis** and anastomosis infection is another well recognized concern during the early post-transplant period<sup>84,92,93</sup>.

**Pneumocystis pneumonia** is rare in the early period following lung transplantation due to the generalized use of chemoprophylaxis during this period of heavy immunosuppressive treatment. However, prophylaxis regimens differ across transplant centers, and studies have shown an increased incidence of late-onset PCP among patients no longer on chemoprophylaxis<sup>94,95</sup>.

## 1.7 Microbiologic diagnostics

Laboratory methods for diagnosing IFD rely on conventional microbiologic methods, *i.e.* **direct microscopy** and **culture** with subsequent species identification, and non-culture methods including **immunological antigen assays**, **enzymatic assays** and molecular methods such as **polymerase chain reaction (PCR)**. Microbiologic methods for fungal detection are afflicted by various shortcomings which will be mentioned in the following sections.

### 1.7.1 Conventional methods

#### 1.7.2 Direct microscopy

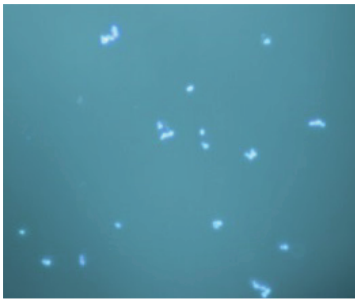
Direct microscopic examination is a crucial first-line procedure in the diagnosis of tissue invasive fungal disease and constitutes a rapid and cost-effective diagnostic method. Fresh clinical samples such as tissue obtained by biopsy, sputum, BAL fluid or sinus aspirate are stained with the fluorescent reagent Calcoflour white or Blankophor and observed directly under fluorescence microscope. The fluorescent stain binds to chitin in the cell wall of the fungus and allows for detection of fungal elements such as budding yeast cells, hyphae or pseudohyphae. Direct microscopic



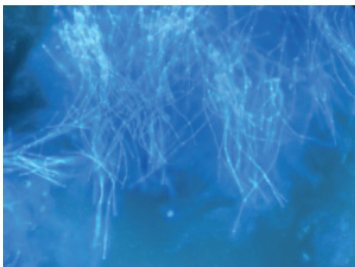
examination thus yields preliminary information on the presence of a **yeast** or a **mold**<sup>2,6,96</sup>. The major advantages of direct microscopy are the rapid means of fungal detection compared to culture and the ability to detect fungal species that are difficult to culture *in vitro* (i.e. *Mucorales*); however, a major drawback of direct microscopy is a limited sensitivity and the inability to differentiate between different fungal species<sup>96</sup>.

For microscopic detection of *Pneumocystis jirovecii* in respiratory specimens, immunofluorescent staining technique is used. The specimen is stained by a fluorescent monoclonal antibody and subsequently examined by fluorescence microscopy. This method is highly specific; however, it is limited by the requirement of well-trained laboratory personnel and by a lower sensitivity compared to PCR-methodology<sup>97</sup>.

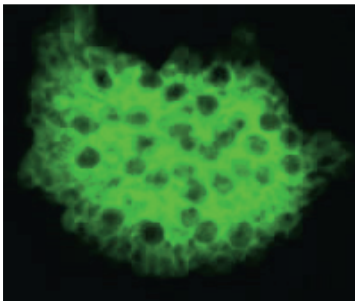
Images of fluorescent microscopy of clinical respiratory tract specimens are shown below:



Specimen stained by Blankophor revealing budding cells of a **yeast**.  
*Courtesy of the Mycology unit at the Department of Clinical Microbiology, Sahlgrenska University Hospital.*



Specimen stained by Blankophor revealing septate hyphae of a **mold**.  
*Courtesy of the Mycology unit at the Department of Clinical Microbiology, Sahlgrenska University Hospital.*



Specimen stained by monoclonal antibodies revealing cystic forms *Pneumocystis jirovecii*. *Public Health Image Library, CDC. Creative Commons Lic.*

### 1.7.3 Cultures

Fungal cultures constitute the corner-stone in the diagnosis of fungal disease since they allow for subsequent species identification and antifungal susceptibility testing. In order to maximize the yield of fungal cultures and allow for growth of both yeasts and molds, clinical specimens are typically cultured on different **enriched selective culture media** (e.g. Sabouraud dextrose agar and Malt extract agar) and **at different incubation temperatures**. Although some fungal species are rapid growing, the turn-around time for fungal growth is usually longer than for bacterial growth, and fungal cultures may require several **weeks of incubation**<sup>2,6,96</sup>.

Fungi may grow in **blood cultures** collected from patients with fungemia after prolonged incubation. Although most fungal species theoretically can be recovered in blood cultures<sup>96</sup>, *Candida* is the predominant fungal species isolated from blood cultures. Blood culture vials with fungal selective growth media, may improve sensitivity for detection of *Candida* spp. in cases with concomitant bacteremia and may shorten the time to positivity for certain *Candida* spp<sup>98</sup>. Although studies suggest that blood cultures are highly sensitive at detecting circulating viable *Candida* cells, *Candida* translocation across the gut mucosa may result only in a transient candidemia and a rapid elimination of *Candida* cells from the circulation which may result in negative blood cultures in patients with deep seated candidiasis<sup>99</sup>. In fact, different studies of autopsy-proven invasive candidiasis have shown that blood cultures collected ante mortem were positive in only 20-70% of the patients, and the sensitivity of blood cultures for the diagnosis of invasive candidiasis overall is low<sup>99</sup>. *Fusarium* and *Cryptococcus* are other medically important fungi that may be recovered from blood cultures; however, the clinical use of blood cultures for the diagnosis of invasive aspergillosis is low. In one study including 91 patients with autopsy proven invasive aspergillosis with concomitantly collected blood cultures, *Aspergillus* fungemia was detected in only one patient<sup>100</sup>, and in another study of 19 HSCT recipients with blood cultures positive for *Aspergillus*, only one had a clinical definition of invasive aspergillosis<sup>101</sup>.

For the diagnosis of tissue invasive fungal disease, clinical **biopsy specimens** from sterile sites are required since fungi recovered from non-sterile sites may represent colonization rather than disease. However, the sensitivity of fungal cultures of biopsy specimens is limited, which may partly result from challenges in identifying optimal sampling sites or uneven distributions of viable organisms in the infected tissue<sup>99</sup>. Furthermore, the

invasive technique required to obtain a biopsy specimen carries a risk for the patient and may be precluded by underlying conditions.

#### 1.7.4 Species identification

There are several routinely available methods for the identification of fungi recovered from clinical specimens. *Candida* spp. may be identified by macroscopic and microscopic **morphologic features** after growth on specialized media (e.g. *Candida* CHROM agar) as well as by **biochemical testing**. Molds may also be identified based on microscopic and macroscopic morphological characteristics. During the last years, however, matrix-assisted laser desorption ionization-time of flight (**MALDI-TOF**) mass spectrometry has emerged as a rapid and precise technique for identification of fungi and is now a routine method for species identification in most microbiologic laboratories<sup>102</sup>. Furthermore, molecular sequencing approaches are increasingly used for species identification of those fungi that are included in the existing sequence databases<sup>96</sup>.

#### 1.7.5 Non-culture diagnostic methods

Considering the above mentioned limitations of conventional microbiologic methods, studies focusing on the utility of non-culture diagnostic methods of fungal cell wall components as antigenic diagnostic markers emerged in the 1980s. Among these markers, the *Aspergillus* antigen galactomannan and the *Candida* antigen mannan were initially most intensely studied<sup>103</sup>. Later on, an enzymatic assay aimed to detect the panfungal cell wall component 1,3- $\beta$ -D-glucan was developed.

**In the papers of this thesis**, assessment of the clinical utility of some non-culture diagnostic methods is included. More detailed description of their methodology can be found in the Methods section 3.3. The work with this thesis and the setup of the prospective studies (papers II and IV) was initiated in 2011. In the following section, the knowledge of non-culture diagnostic methods present at the time of the initiation of this thesis will be described. Up to date knowledge on the utility of some of these methods for the diagnosis of IFD in immunocompromised hosts will be further explored in the Results & Discussion section and in the four included papers.

#### 1.7.6 Galactomannan

Galactomannan, a cell-wall polysaccharide specific to *Aspergillus* spp. was first detected in body fluids of experimental animals and patients with **invasive aspergillosis** in the 1980s<sup>104,105</sup>. Galactomannan was included as a

microbiologic criterion for invasive aspergillosis in the 2002 definitions for invasive fungal infections by the European Organization for Research and Treatment of Cancer and Mycoses Study Group (EORTC/MSG)<sup>106</sup>. In 2003, a commercial enzyme immunoassay (EIA) to detect galactomannan in **serum** and **BAL** fluid (Platelia *Aspergillus* EIA test, Bio-Rad Laboratories) was approved by the U.S. Food and Drug Administration for use in the diagnosis of invasive aspergillosis in HSCT recipients and in patients with leukemia.

In a meta-analysis published in 2006, the authors reported that the diagnostic accuracy of galactomannan for the diagnosis of invasive aspergillosis was overall moderate, but higher in patients with high-risk hematologic disorders than in solid organ transplant recipients. The studies included in the meta-analysis, however, displayed a large heterogeneity<sup>107</sup>.

### 1.7.7 Mannan and anti-mannan

Mannan, a major component of the *Candida* cell wall can be detected in **serum** during invasive infection. Enzyme immunoassays for the detection of mannan or anti-mannan antibodies were developed in the late 1990s<sup>108</sup> and are readily accessible in some European countries. Studies have shown a moderate sensitivity of mannan for the diagnosis of invasive candidiasis; however if combining mannan with the detection of anti-mannan antibodies, the diagnostic accuracy was approved<sup>109</sup>.

### 1.7.8 1,3-β-d-glucan

A method for measuring 1,3-β-D-glucan (betaglucan) in **serum** was established in Japan as a diagnostic test for **IFD** in the early 1990s<sup>110,111</sup>. As previously described, 1,3-β-D-glucan is a major cell wall component of most medically important fungi with the exception of *Cryptococcus* and fungi belonging to *Mucorales* that have no or low levels of 1,3-β-D-glucan in their cell walls. Three assays for betaglucan testing are currently available in Japan (Fungitec<sup>®</sup>, Wako<sup>®</sup> and Maruha<sup>®</sup>) and the Fungitell<sup>®</sup> assay, a slight modification of the previously developed Glucatell assay (Associates of Cape Cod, MA, USA), was approved by the U.S. Food and Drug Administration in 2004. In 2008, betaglucan was included as a mycological factor in the revised diagnostic criteria of the EORTC/MSG<sup>112</sup>. In 2009, a large retrospective study was published which reported a sensitivity and specificity of 64 and 84%, respectively, among 112 patients with mixed types of proven or probable IFDs<sup>113</sup>. Two meta-analyses published in 2011 concluded that betaglucan seemed to be a useful fungal diagnostic fungal marker, but that the results should be interpreted with caution<sup>114,115</sup>.

## 1.7.9 Others

### D-arabinitol

D-arabinitol is a metabolite produced by most medically important *Candida* spp. with the exception of *C. krusei*. In 1979, D-arabinitol was detected in serum from patients with invasive candidiasis<sup>116</sup>, and in the 1990s, a method to detect D-arabinitol by gas chromatography in serum and urine samples was developed<sup>117</sup>. D- and L-arabinitol are also present in human serum and urine, and a method based on the measurement of the D-/L-arabinitol (DA/LA) ratio in urine samples showed higher specificity<sup>118</sup>. The measurement of DA/LA ratio in serum or urine samples was consequently proposed as a diagnostic marker for invasive candidiasis<sup>119</sup> although large studies evaluating its implementation in the clinical diagnosis of invasive candidiasis were lacking<sup>120</sup>.

### Bis(methyl)gliotoxin

Gliotoxin, a metabolite produced by *Aspergillus* spp. during invasive growth was detected in sera of patients with invasive aspergillosis and proposed as a possible marker of IA<sup>121,122</sup>. Bis(methyl)gliotoxin (bm-gliotoxin), the inactive metabolite of gliotoxin, was later on presented as a more stable and reliable marker for IA<sup>123</sup>. A method for detection of bm-gliotoxin in serum by using high-performance thin-layer chromatography (HPTLC) with ultra-violet (UV) as well as mass spectrometric (MS) detection was published in 2012 and proposed as a possible diagnostic tool for IA<sup>123</sup>.

## 1.7.10 PCR

Sensitive and specific real-time PCR methodology revolutionized the field of microbiologic diagnostics in the 2000s and is now widely used for pathogen detection in microbiology laboratories. PCR assays specific for *Pneumocystis jirovecii* were introduced in the early 2000s and proved to be very sensitive for the diagnosis of *Pneumocystis* pneumonia in immunocompromised hosts<sup>124</sup>. However, positive *Pneumocystis* PCR was also found in patients with no clinical signs of pneumonia and it was suggested that while a negative *Pneumocystis* PCR in a BAL fluid specimen could be used to rule out PCP, a positive PCR result should only be interpreted in parallel with clinical findings<sup>125,126</sup>.

The use of PCR assays for the detection of other medically important fungi, such as *Aspergillus* and *Candida* has not reached the same level of acceptance as PCR assays for *Pneumocystis* detection. Although extensive

work has been conducted, including the European *Aspergillus* PCR initiative, a working group of the International Society for Human and Animal Mycology organized in 2006 with the aim of providing optimized, standardized laboratory protocols for implementation of *Aspergillus* PCR in the clinical routine, there was still no consensus on the diagnostic utility of PCR methodology for the detection of *Candida* or *Aspergillus* during the time of the work with this thesis<sup>127,128</sup>.

## 2 AIMS

The overall aim of this thesis was to gain greater understanding of the spectrum of invasive fungal disease and, in particular, to explore the role of different fungal markers and microbiological assays for the diagnosis of IFD in different cohorts of immunocompromised hosts.

The specific aims were:

- To investigate and explore the diagnostic accuracy of betaglucan for the diagnosis of IFD and possible confounding factors to positive test results in patients with hematologic disorders
- To investigate the role of betaglucan in combination with other diagnostic fungal markers (galactomannan, DA/LA ratio and bis(methyl)gliotoxin) in a high-risk hematology setting
- To investigate whether or not serum sample based diagnosis of *Pneumocystis* pneumonia using *Pneumocystis* PCR and betaglucan may be feasible in HIV-infected patients
- To investigate the long-term clinical impact of fungal colonization and tracheobronchitis in lung transplant recipients
- To investigate the role of betaglucan as a diagnostic marker for fungal tracheobronchitis after lung transplantation

## 3 PATIENTS & METHODS *with discussion*

### 3.1 Patient populations, study designs & ethics

All four papers of this thesis included adult immunocompromised patients with risk for developing IFD. Cohorts with different underlying immunosuppressive disorders and with risk for different types of IFD were studied. Papers I-III were primarily diagnostic studies but with different study designs. Paper IV included a diagnostic part although the principal aim was to study the clinical impact of fungal disease.

**Paper I** and **paper II** included patients with hematologic malignancies and alloHSCT recipients.

- **Paper I** was a *retrospective cohort study* with the following inclusion criteria: Adult patients with hematologic disorders admitted to the Department of Hematology at Sahlgrenska University Hospital in Gothenburg during the period 2009–2011 who had at least two consecutive beta-glucan tests performed during this period.
- **Paper II** was a *prospective multicenter cohort study* with the following inclusion criteria: adult patients hospitalized due to newly diagnosed acute leukemia, myelodysplastic syndrome or high-grade malignant lymphoma intended for curative treatment; newly diagnosed aplastic anemia; or alloHSCT at Sahlgrenska University Hospital in Gothenburg and Skåne University Hospital in Lund between September 2011 and December 2012. Blood samples were collected once to twice weekly and urine samples and fungal surveillance cultures were collected once weekly during 3 months following inclusion and at all subsequent episodes of hospitalization during the 18-months study period.
- **Paper III** was a *retrospective diagnostic case-control study* with the following inclusion criteria: HIV-infected patients who had received a diagnosis of PCP at Sahlgrenska University Hospital in Gothenburg between 2005 and 2018 and who had a frozen serum sample that had been collected within 5 days prior to the start of PCP treatment. HIV-



infected patients matched for blood CD4+ cell counts without a clinical diagnosis of PCP and blood donors were included as negative control subjects.

- **Paper IV** was a *prospective nationwide cohort study* with the following inclusion criteria: adult Swedish patients who were accepted for lung transplantation at Sahlgrenska University Hospital in Gothenburg and Skåne University Hospital in Lund during 2012-2014. BAL fluid and serum samples were collected systematically during the first year post-transplant and evaluated for fungal cultures (BAL) and betaglucan (BAL and serum). Clinical follow-up was conducted until December 2018.

The types of IFD studied in the different papers were: **mixed IFD** (papers I and II), **PCP** (paper III) and *Aspergillus* and *Candida tracheobronchitis* (paper IV).

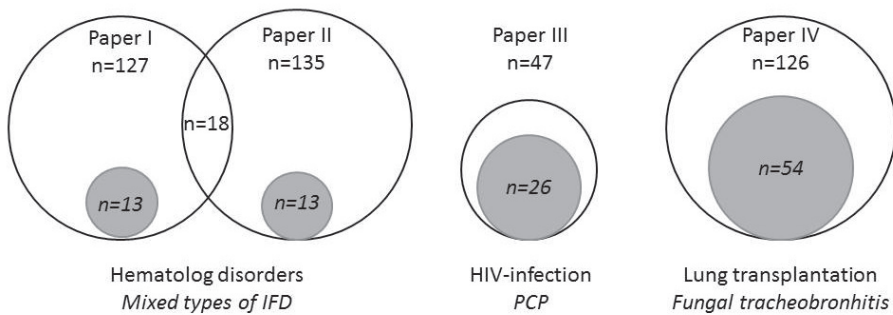


Figure 11. Diagram showing the relative sizes of the study populations in each paper, and the number of overlapping patients in paper I and II (out of which one was a case of IFD). The gray circles represent the number of patients with proven or probable IFD in each study.

All studies were approved by the Regional Ethics Committee of Gothenburg (Dnr 433-08, 959-13 and 735-18). Informed written consent was obtained from all patients included in the two prospective studies (papers II and IV).

## 3.2 Definitions

### 3.2.1 Defining 'IFD'

#### EORTC/MSG

In an attempt to facilitate harmonization of clinical and epidemiological studies in the field of IFD, a consensus group of the EORTC/MSG group published standard definitions for invasive fungal disease in 2002<sup>106</sup> developed for patients with cancer and for HSCT recipients. The definitions were subsequently revised in 2008<sup>112</sup> and extended to include other immunocompromised patients such as solid organ transplant recipients. The definitions include three levels of probability to the diagnosis of IFD: proven, probable and possible. Proven IFD requires the detection of fungal elements in a clinical specimen collected from a sterile site, whereas probable IFD relies on specific host factors, typical clinical criteria and mycological evidence, and possible IFD relies on specific host factors and typical clinical criteria, but no mycological evidence (Table 1).

#### ISHLT

In 2010, the International Society for Heart and Lung Transplantation (ISHLT) published consensus-derived expert opinion of definitions for infections in cardiothoracic transplant recipients, including definitions for invasive fungal disease<sup>85</sup>. The authors point out that although the revised 2008 EORTC/MSG definitions were extended to also include organ transplant recipients, they may still not be fully applicable to lung transplant recipients due to the unique characteristics of fungal disease seen in this patient group. Some examples that are highlighted are differences in radiological findings in invasive aspergillosis and differences in diagnostic accuracy of the indirect tests (included as mycological evidence in the EORTC/MSG criteria) in lung transplant recipients compared to patients with hematologic disorders.

Table 1. The EORTC/MSG criteria for the definition of IFD<sup>112</sup>

<b>Proven</b>	<b>Fungi detected by microscopy or recovered by culture from a sterile site</b>
<b>Probable</b>	Presence of a <b>host factor</b> , a <b>clinical criterion</b> and a <b>mycological criterion</b>
<b>Possible</b>	Presence of a <b>host factor</b> and a <b>clinical criterion</b>
<b>Host factors</b>	
<ul style="list-style-type: none"> <li>• Neutropenia (<math>&lt;0.5 \times 10^9</math> neutrophils/L for <math>&gt;10</math> days temporally related to the onset of IFD)</li> <li>• Receipt of an allogeneic stem cell transplant</li> <li>• Corticosteroids (mean minimum dose of 0.3 mg/kg/day of prednisone equivalent for 13 w)</li> <li>• Treatment with T cell immunosuppressants, such as cyclosporine, TNF-<math>\alpha</math> blockers, specific monoclonal antibodies or nucleoside analogues during the past 90 days.</li> <li>• Inherited severe immunodeficiency</li> </ul>	
<b>Clinical criteria</b>	
<p><b>Lower respiratory tract fungal disease</b>  <i>The presence of 1 of the following 3 signs on CT:</i></p> <ul style="list-style-type: none"> <li>• Dense, well-circumscribed lesions(s) with or without a halo sign</li> <li>• Air-crescent sign</li> <li>• Cavity</li> </ul> <p><b>Tracheobronchitis</b></p> <ul style="list-style-type: none"> <li>• Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis</li> </ul> <p><b>Sinonasal infection</b>  <i>Imaging showing sinusitis plus at least 1 of the following 3 signs:</i></p> <ul style="list-style-type: none"> <li>• Acute localized pain (including pain radiating to the eye)</li> <li>• Nasal ulcer with black eschar</li> <li>• Extension from the paranasal sinus across bony barriers, including into the orbit</li> </ul> <p><b>CNS infection</b>  <i>1 of the following 2 signs:</i></p> <ul style="list-style-type: none"> <li>• Focal lesions on imaging</li> <li>• Meningeal enhancement on MRI or CT</li> </ul> <p><b>Disseminated candidiasis</b>  <i>At least 1 of the following 2 entities after an episode of candidemia within the previous 2 weeks:</i></p> <ul style="list-style-type: none"> <li>• Small, target-like abscesses (bull's-eye lesions) in liver or spleen</li> <li>• Progressive retinal exudates on ophthalmologic examination</li> </ul>	
<b>Mycological criteria</b>	
<p><b>Direct test (cytology, direct microscopy, or culture)</b>  <i>Mold in sputum, bronchoalveolar lavage fluid, bronchial brush, or sinus aspirate samples, indicated by 1 of the following:</i></p> <ul style="list-style-type: none"> <li>• Presence of fungal elements indicating a mold</li> <li>• Recovery by culture of a mold (e.g., Aspergillus, Fusarium, Zygomycetes etc.)</li> </ul> <p><b>Indirect tests (detection of antigen or cell-wall constituents)</b>  <i>Aspergillosis</i></p> <ul style="list-style-type: none"> <li>• Galactomannan antigen detected in plasma, serum, bronchoalveolar lavage fluid, or CSF</li> <li>• Aspergillus PCR in blood or BAL fluid <u>new proposal, not included in 2008</u></li> </ul> <p><i>Invasive fungal disease other than cryptococcosis and zygomycoses</i></p> <ul style="list-style-type: none"> <li>• <math>\beta</math>-d-glucan detected in serum</li> </ul>	

### **Some notations about the consensus definitions of IFD**

In the field of cardiothoracic transplantation, many studies dealing with infections, published in high impact journals, use the ISHLT definitions to define cases of fungal disease. In an attempt to adapt the clinical criteria to the pulmonary manifestations seen in this specific patient group, the ISHLT working group omitted the requisite of specific CT-based radiological findings included in the EORTC/MSG definitions. Instead, the following finding on a plain chest radiograph: ‘new or progressive and persistent infiltrate’ was accepted as a radiological criterion<sup>85</sup>. However, for a recently transplanted patient, perhaps still admitted to the intensive care unit after extensive surgery, the low specificity of this criterion must be recognized. Although this definition may facilitate the enrollment of eligible patients with IFD in clinical studies, it introduces a bias towards the inclusion of dubious cases. This is a factor that needs to be considered when interpreting results of diagnostic accuracy in studies employing these definitions.

In contrast, in the 2008 updated version from the EORTC/MSG consensus group, the definitions of IFD were revised specifically to lower the risk of including dubious cases such as those with nonspecific pulmonary infiltrates<sup>112</sup>. These definitions thus yield a more well-defined study population, which may result in more reliable results of *e.g.* diagnostic accuracy studies. On the other hand, the stringent definition may limit the number of eligible patients for such studies.

**In this thesis, the EORTC/MSG criteria were used** to define IFD caused by yeasts or molds throughout **all papers**. We chose to use these definitions since they are adopted by the scientific research community in the field of IFD as the ‘gold standard’ for the definition of IFD. We chose to use these definitions also for the study on lung transplant recipients in order to have conformity throughout the thesis and in order to obtain a more stringent classification of cases.

### **3.2.2 Defining PCP**

An important limitation to both the EORTC/MSG and ISHLT definitions of fungal disease is that none include a definition for *Pneumocystis pneumonia*. Although there is no published set of standard definitions for PCP most clinical studies employ a definition for PCP based on host factors, microbiological and clinical criteria. Originally, the microbiological criterion for a case of PCP consisted of the detection of *P jirovecii* by microscopy and immunofluorescent staining; however, later publications also include a

positive *Pneumocystis* PCR from a respiratory sample as a microbiological criterion for PCP.

In paper III, we used the following previously described<sup>129</sup> definition for PCP:

**Host factor**

- HIV infection

*and*

**Microbiological criterion**

- *Pneumocystis jirovecii* detected by PCR and/or microscopy with immunofluorescent staining in a sputum or BAL specimen

*and*

**Clinical criteria** (*a minimum of two out of the following three*):

- respiratory symptoms consisting of cough and/or dyspnea
- hypoxia <95%
- typical radiological picture such as ground glass opacity on chest CT scan or diffuse interstitial opacity on chest x-ray

*and*

Receipt of a full course of treatment for PCP

*and*

No other alternative etiology to the clinical picture

## **3.3 Laboratory methods**

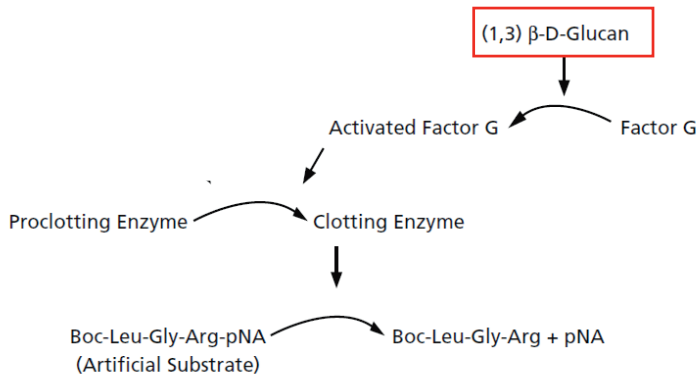
In all papers of this thesis, microbiological diagnostic methods of fungal infections were included.

### **3.3.1 1,3-β-D-glucan**

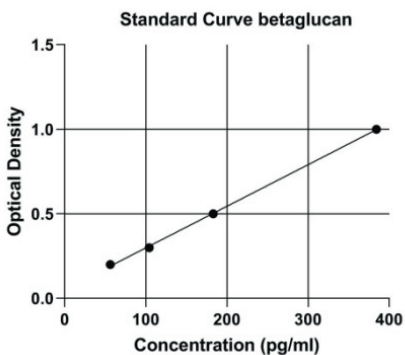
**All four papers** in this thesis included the analysis of betaglucan.

The Glucatell<sup>®</sup> assay kit (Ass. Cape Cod, MA, USA) was used for measuring levels of betaglucan at the Department of Clinical Microbiology at Sahlgrenska University Hospital. The betaglucan analysis is based on an enzymatic colorimetric reaction between Factor G in the assay reagent (obtained from a lysate of the amoebocyte blood cell from the horseshoe crab *Limulus Polyphemus*) and 1,3-β-D-glucan in the sample. 1,3-β-D-glucan activates Factor G which in turn activates a cascade of enzymatic reactions.

The final step in the reaction includes a chromogenic substrate permitting spectrophotometric quantitation of the product of the reaction:



Serum samples were pretreated with heat at 75°C for 10 minutes and analyzed according to the manufacturer’s instructions. In short, GlucateLL<sup>®</sup> reagent was added to vials of patient sera (in duplicate), internal negative and positive controls and standard solutions with known betaglucan concentrations (in triplicate). The samples were incubated at 37°C during the process of the enzymatic reaction. The optical density of each sample was then measured with a spectrophotometer. A betaglucan concentration for each patient sample was calculated from the standard curve of the known betaglucan concentrations. The lowest and highest limit of detection of the GlucateLL<sup>®</sup> assay is 50 and 400 pg/ml, respectively. The following cut-offs are recommended by the manufacturer to define test results: <60 pg/ml: negative, 60-80 pg/ml: indeterminate, >80 pg/ml: positive.



*A standard curve for the betaglucan GlucateLL<sup>®</sup> assay based on standard solutions with known betaglucan concentrations. The betaglucan concentration is plotted as a function of the optical density.*

**In papers I and IV**, the betaglucan levels were reported as the values between the limits of detection (50-400 pg/ml). For calculations of diagnostic

accuracy in paper I, the betaglucan values below or above the limits of detection were converted to 50 and 450 pg/ml, respectively.

**In paper II**, stored serum samples previously yielding betaglucan levels above the maximum level of detection were retrieved, diluted 1:5 and 1:10 with glucan-free reagent grade water from the GlucateLL<sup>®</sup> assay kit and reanalyzed in order to obtain an exact value of betaglucan concentration.

**In paper III**, all samples yielding betaglucan values >400 pg/ml in the first round of analysis were diluted 1:5 and reanalyzed to obtain a maximum level of detection of 2000 pg/ml.

**In paper IV**, betaglucan concentrations were also measured in BAL fluid samples. The BAL fluid was centrifuged, and aliquots of the supernatant were pretreated and analyzed according to the protocol for serum samples.

### 3.3.2 Other laboratory methods

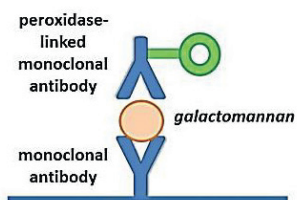
In addition to the analysis of betaglucan, **paper II** also included the following diagnostic analyses or methods:

#### 3.3.3 *Candida* surveillance cultures

Clinical specimens from urine, rectum and oral cavity were consecutively cultured on Sabouraud dextrose and *Candida* CHROM agar plates at the Department of Clinical Microbiology in Gothenburg. Isolated yeasts were identified to the species level using MALDI-TOF and morphological criteria. The recovery of culture was roughly quantified as sparse, moderate or abundant growth.

#### 3.3.4 Galactomannan

Galactomannan levels in serum were measured using the Platelia<sup>™</sup> *Aspergillus* EIA assay (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's instructions at the Department of Clinical Microbiology at Sahlgrenska University Hospital. The galactomannan assay is a one-stage immunoenzymatic sandwich assay using microplate wells coated with monoclonal antibodies directed against galactomannan in clinical specimens. The optical density of each sample is measured with a spectrophotometer and the result is obtained as an index of the optical density of the clinical sample and a cut-off control serum (ODI). The following cut-offs are recommended by the manufacturer to define test results: <0.5 ODI: negative, ≥0.5 ODI: positive.



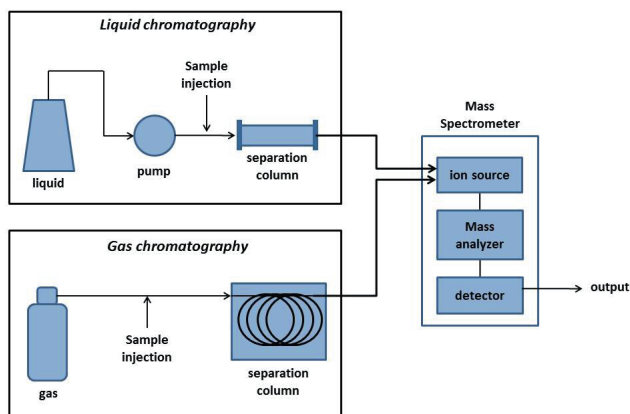
*A schematic image of the galactomannan immunoenzymatic sandwich assay.*

### 3.3.5 Bis(methyl)gliotoxin

Bm-gliotoxin in serum was analyzed using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) similar to previously published methodology<sup>123</sup>. The analysis was performed at the Department of Laboratory Medicine at the University of Lund. Liquid chromatography methodology was used for the separation of bm-gliotoxin in the sample by pumping pressurized liquid solvent containing the sample mixture through a liquid column where different molecules are separated. Bm-gliotoxin in the sample was then detected by mass spectrometry. For detailed methodology, see paper II (Supplementary).

### 3.3.6 DA/LA ratio in urine

DA/LA ratio in urine samples was analyzed using gas chromatography mass spectrometry (GC-MS) according to previously described methodology<sup>130</sup>. The analysis was performed at the Department of Laboratory Medicine at the University of Lund. In short, urine samples previously administered to filter papers were extracted with methanol and pretreated as previously described<sup>130</sup>. DA and LA were then separated using gas chromatography and detected by mass spectrometry. The ratio of DA/LA was calculated. A ratio  $>5$  was considered positive, 3-5 indeterminate and  $< 3$  negative.



*Schematic picture of liquid chromatography and gas chromatography with mass spectrometry.*



### 3.3.7 *Pneumocystis* PCR

In **paper III**, a real time *Pneumocystis* PCR assay was used to detect *Pneumocystis* DNA in serum samples. The PCR-assay is an in house assay developed at the Department of Clinical Microbiology at Sahlgrenska University Hospital, based on a slight modification of the primers and probes targeting the mitochondrial gene coding for the large ribosomal subunit (mtLSU) of *P. jirovecii* previously described by Dini *et al.*<sup>131</sup>. The PCR analysis was performed using the automated BD MAX system (BD Diagnostics, Franklin Lakes, New Jersey, US) which includes lysis of samples, DNA-extraction and real-time PCR. The real-time PCR reaction allowed for amplification of DNA during 46 cycles. The cycle threshold (Ct) value obtained from a positive PCR result was used to represent the amount of *Pneumocystis jirovecii* DNA in the sample. Detailed methodology is described in paper III. This PCR-assay is a certified analysis for respiratory specimens at the Department of Clinical Microbiology at the hospital.

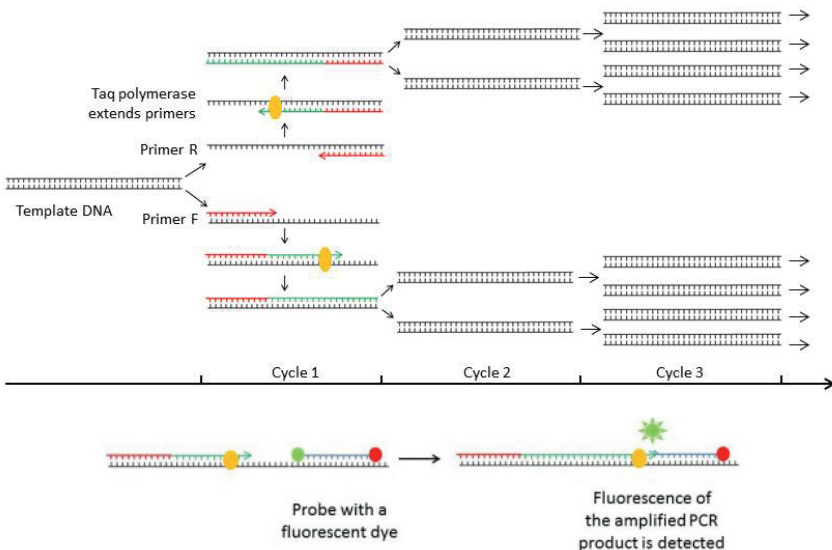


Figure 12. **Top:** The principle of a PCR reaction. 1. The template DNA is denatured at 96°C. 2. The temperature is lowered and the reverse and forward primers bind to the flanks of the target region (annealing). 3. The temperature is elevated to approx. 70°C so that the Taq-polymerase can extend the primers and synthesize new strands of DNA (one cycle). This results in an exponential amplification of PCR-products. **Bottom:** In real-time PCR, a probe labeled with a fluorescent dye is included in the reagent mix. The probe binds to a specific sequence of the template DNA and the fluorescent dye is activated when the extension of the template DNA is completed. The amount of measured fluorescence thus reflects the amount of amplified DNA.

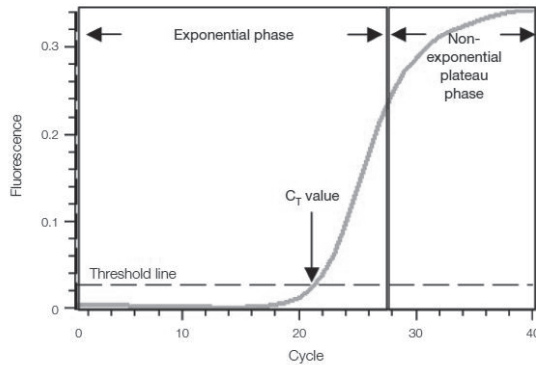


Figure 13. The amplification curve in real-time PCR. The amount of detectable fluorescent signal (y-axis) increases as the cycles of the PCR-reaction are proceeding (x-axis). The pattern of the curve reflects the exponential increase of amplified DNA. As the reaction components of the PCR-assay are being consumed, the reaction slows and finally enters a plateau phase. The cycle number at which enough amplified DNA has accumulated to yield a detectable fluorescent signal is called the cycle threshold ( $C_t$ ) value. This value reflects the initial amount of template DNA present in the sample.

## 3.4 Statistical methods

A significance level of 95% was accepted for statistical hypothesis testing throughout this thesis.

### 3.4.1 COMPARISON BETWEEN GROUPS

#### 3.4.2 Univariate comparison between groups

In all **papers of this thesis**, univariate comparison between groups was done using non-parametric tests due to skewness of data and, in some cases, small sample sizes.

**Comparison between two groups:** The Mann Whitney U test was used to compare medians and the Fisher's exact test to compare proportions (**papers I, II, III**).

**Comparison between multiple groups:** In **paper IV**, the Kruskal Wallis test was used to compare medians. *Post-hoc* analysis to evaluate which groups differed from the others was done by Dunn's multiple comparison test.

### 3.4.3 Multivariate analysis

In **paper I**, multivariate analysis was done to investigate potential confounding factors to positive betaglucan results among patients with no IFD. Due to repeated measures of betaglucan per patient, the first step consisted of a bivariate analysis of variance including the variable of interest and study subject as a covariate. The variables that were statistically significant in the bivariate analysis were then included in a multiway analysis of variance model.

### 3.4.4 Correlation analysis

Correlation analysis is used to assess whether or not there is a linear association between two continuous variables. A correlation coefficient of  $\pm 1$  indicates a perfect linear association (directly or inversely related), while 0 indicates no linear association.

In **papers II and III**, Spearman's correlation coefficient was used to assess correlation. This is a non-parametric method that may be used in a scenario with small sample sizes or skewed data. In a situation when measured variable has clear limits of detection, such as the case of betaglucan levels in serum, the outcome cannot follow a normal distribution, why a non-parametric method may be preferable to use<sup>132</sup>.

### 3.4.5 Evaluation of diagnostic accuracy

A recurrent theme in this thesis is the evaluation of diagnostic accuracy of fungal microbiological markers or assays for the diagnosis of IFD. **Papers I-IV** all included analyses of diagnostic accuracy.

The diagnostic accuracy of a test to detect a specific disease (the target condition) is dependent upon the definition of the target condition against which the test is being evaluated. The means by which the target condition is defined in a study is referred to as the **reference standard** of the condition. The reference standards used for analysis of diagnostic accuracy were as follows:

- Papers I-II** Proven or probable IFD according to EORTC/MSG criteria
- Paper III** PCP according to previously described definition
- Paper IV** Probable tracheobronchitis according to EORTC/MSG criteria with a modification to include *Candida* spp.

**Papers I-III** included reports of estimates of the sensitivity, specificity, positive and negative predictive value (PPV and NPV) of the test being evaluated. These estimates are calculated by cross tabulation, or two-by-two tables:

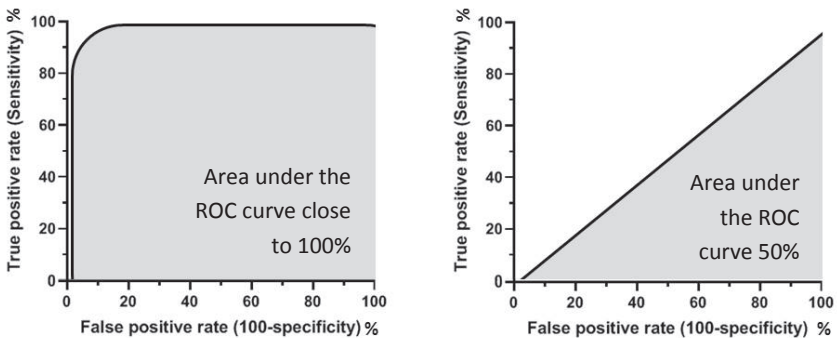
		<b>target disease (reference standard)</b>	
		Present (n)	Absent (n)
<b>index test</b>	Positive (n)	True positive (TP)	False positive (FP)
	Negative (n)	False negative (FN)	True negative (TN)

- Sensitivity**      $TP / (TP+FN)$   
**The fraction of those with the disease correctly identified as positive by the test**  
*-or, the probability that the test is positive given a patient has the disease*
- Specificity**      $TN / (FP+TN)$   
**The fraction of those without the disease correctly identified as negative by the test**  
*-or, the probability that the test is negative given a patient does not have the disease*
- PPV**      $TP / (TP+FP)$   
**The fraction of those with positive tests who actually have the disease**  
*-or, the probability that a patient with abnormal test results is truly abnormal*
- NPV**      $TN / (FN+TN)$   
**The fraction of those with negative tests who actually don't have the disease**  
*-or, the probability that a patient with normal test results is truly normal*

As can be inferred from the cross tabulation shown above, the predictive values depend on the prevalence of the disease of interest in the study population, or, on the **pre-test probability** of disease ( $TP+FN$ /total number of individuals). In **Papers I and III**, the estimates of predictive values were calculated at expected prevalence rates of IFD and based on the obtained estimates of sensitivity and specificity, according to Bayes' Theorem.

Receiver operating characteristics (ROC) curve is another method for presenting the diagnostic accuracy of a specific test. This method was used in **Papers I, III and IV.**

In a ROC curve, the true positive rate (sensitivity) is plotted as a function of the false positive rate (100-specificity) for different cut-off points of the test being evaluated. Each point on the ROC curve thus represents a sensitivity/specificity pair corresponding to a particular cut-off level. The area under the ROC curve gives an overall measure of the ability of the test to discriminate individuals with the disease of interest from individuals without the disease. ROC curves are typically used to determine the optimal cut-off level for the test of interest<sup>133</sup>.



*Figure 14. The figure to the left shows a ROC curve of a test where the diagnostic accuracy is close to perfect, i.e. the test has a nearly 100% ability to discriminate individuals with the disease from individuals without the disease. The figure to the right shows a ROC curve of a test with no ability to discriminate disease from no disease, i.e. a test where the discrimination ability is no better than chance.*

### **3.4.6 Survival analysis**

**In paper IV,** survival analysis methods (Kaplan-Meier and Cox regression) were used to assess the clinical impact of fungal colonization and tracheobronchitis after lung transplantation.

Survival analyses are methods for assessing the effect of a certain predictor on a specific event, or outcome, during a specified time period, taking into account the time to the specific event. Subjects for whom the event did not occur are typically treated as censored subjects. Censoring may occur before the end of the observation time (*i.e.* the event of interest cannot be

determined due to *e.g.* unrelated death or lost to follow-up) or at the end of the observation time (the event did not yet occur).

Kaplan-Meier estimate is a descriptive method where the time to the event of interest, in the presence of censored subjects, may be compared between different groups using the log-rank test of significance. This method, however, is a univariate method and does not take into account the effect of confounding covariates on the same event.

In order to assess the effect of a certain predictor on the event or outcome of interest in the presence of other covariate predictors, regression analysis may be done. Regression analysis of time-to-event is called Cox regression, or Cox proportional hazards. Cox regression analysis may include one predictor (univariate Cox regression) or multiple covariate predictors (multivariate Cox regression)<sup>134</sup>.

In Paper IV, various “fungal status” predictors (*Section 4.5*) were evaluated with respect to the outcomes ‘Bronchiolitis obliterans syndrome (BOS)’ and death. In the Cox-regression models, unrelated death events were treated as time points for censoring.

The cumulative one-year incidence of fungal colonization or tracheobronchitis was calculated using survival analysis where patients who died before the end of the one-year follow-up time were treated as censored subjects.

## 4 RESULTS *with discussion*

### 4.1 1,3- $\beta$ -D-glucan and diagnostic accuracy

The diagnostic accuracy of betaglucan was addressed in all **four papers** of this thesis; however, patient cohorts, types of IFD and study designs were different. Figure 15 shows the betaglucan levels in all patients with proven or probable IFD included in the four papers, categorized into type of fungal disease and patient cohort.

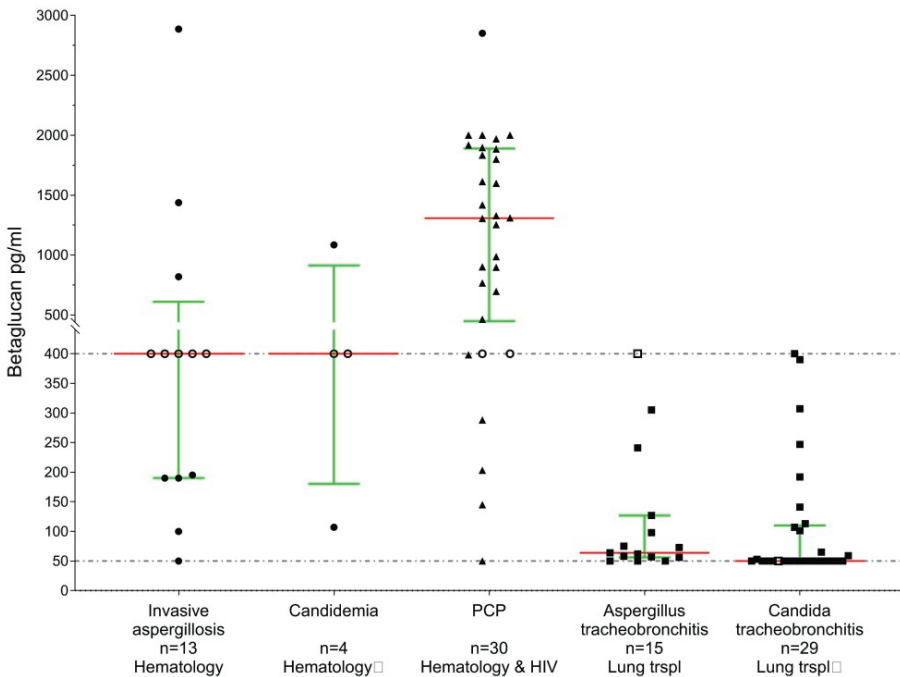


Figure 15. Betaglucan levels in all patients with IFD in papers I-IV. The following betaglucan samples are shown in the graph: Hematology cohort (circles): sample collected within 2 weeks from diagnosis of IFD; HIV-cohort (triangles): sample collected at the time of PCP diagnosis; Lung transplanted cohort: sample collected at the time point of the first positive fungal culture in BAL. Solid bars represent median and interquartile ranges. The dotted lines indicate the minimum and maximum level of detection. Those samples that were not titrated to yield the exact betaglucan level above the maximum level of detection are shown as empty symbols.

### 4.1.1 Hematology cohorts (Papers I and II)

The utility of betaglucan for the diagnosis of IFD in high-risk hematology patients was evaluated in papers I and II. Paper I was an exploratory retrospective study, while paper II was prospectively designed to further investigate the findings of paper I. A total number of 25 patients with proven or probable IFD were included in the two studies. The patients with IFD are presented in Table 2.

### 4.1.2 Sensitivity and specificity

The reported estimates of diagnostic accuracy of betaglucan for the diagnosis of IFD were different in the two papers. The overall diagnostic accuracy of betaglucan in **paper I** was very high with an area under the ROC curve of 0.98 and high estimates of sensitivity and specificity (92 and 96%, respectively) at a cut-off level of 158 pg/ml (*i.e.* the cut-off level yielding the highest estimates of diagnostic accuracy in that study). Although betaglucan sampling was performed in a similar cohort of patients in **paper II**, the reported estimates of diagnostic accuracy were significantly lower in the prospective study (Table 3). Some explanations to this variation in results, which highlight some of the difficulties in designing and interpreting results of diagnostic studies, will now be discussed.

Both papers I and II were longitudinal cohort studies where the patients underwent sequential betaglucan sampling over time. Although longitudinal cohort studies are generally considered superior to case-control studies when evaluating the diagnostic accuracy of a test<sup>135,136</sup>, some important factors need to be identified and considered when interpreting the results of such studies. As exemplified by the different results of papers I and II, the selection of index test sample for calculations of per-patient estimates of sensitivity, specificity and predictive values in studies where patients are sampled over time will have a great impact on the results. This was also highlighted by Marr *et al.* in a paper addressing complicating factors in diagnostic studies of IFD<sup>137</sup>. For patients who develop the disease of interest, the sample collected at the onset of disease may be the most relevant sample to use for calculating the sensitivity of the test; however, the time point of onset of fungal disease may be difficult to assess in this group of patients who often suffer from multiple opportunistic infections and where the reference standard of the target disease may be insensitive or delayed due to the requirement of a CT scan<sup>137</sup>. For patients who do not develop the disease of interest during the sampling period, the selection of index test sample for calculating the specificity of the test is not straight forward and different approaches may be used to overcome the problem of repeated measures.



Table 2. Patients with proven or probable IFD in papers I and II.

IFD	Pat	Paper	Underlying condition	Transplant	Body site infected	Fungal specimen	Outcome
Proven	1	I	AML	-	Blood	<i>C. albicans</i>	†
	2	I	AML	-	Blood & spleen	<i>C. dubliniensis</i>	Survival
	3	I	AML	-	Blood & lungs	<i>C. dubliniensis</i>	Survival
	4	II	ALL	-	Blood & skin	<i>C. krusei</i>	Survival
	5	I & II	AA	alloHSCT	Lungs	<i>A. fumigatus</i>	Lobectomy, survival
	6	I	AA	-	Lungs	<i>A. fumigatus</i> & <i>A. versicolor</i>	†
	7	II	CLL	alloHSCT	Lungs & brain	<i>A. fumigatus</i>	†
	8	I	CLL	alloHSCT	Lungs, skin, brain	<i>Rhizomucor pusillus</i>	†
	9	II	AML	-	Blood & skin	<i>Fusariumverticillioides</i>	Survival
	10	I	AML	alloHSCT	Lungs & liver	Mold, NOS	Survival
Probable	11	I	AA	alloHSCT	Sinus	<i>A. fumigatus</i>	Survival
	12	I	MDS	alloHSCT	Lungs, brain & kidney	<i>A. fumigatus</i>	Survival
	13	I	ALL	alloHSCT	Lungs	<i>A. fumigatus</i>	†
	14	II	CML	alloHSCT	Lungs	<i>A. fumigatus</i>	Survival
	15	II	ALL	alloHSCT	Lungs	<i>A. fumigatus</i>	†
	16	II	AML	alloHSCT	Lungs	<i>A. ustus</i>	Survival
	17	II	AML	-	Lungs	<i>Aspergillus</i> spp.	†
	18	II	Lymphoma	alloHSCT	Lungs & brain	<i>Aspergillus</i> spp.	†
	19	I	ALL	-	Lungs	<i>Aspergillus</i> spp.	Survival
	20	I	CLL	alloHSCT	Lungs	<i>Aspergillus</i> spp.	Survival
	21	I	ALL	alloHSCT	Lungs	<i>P. jirovecii</i>	Survival
	22	II	Lymphoma	-	Lungs	<i>P. jirovecii</i>	†
	23	II	ALL	-	Lungs	<i>P. jirovecii</i>	Survival
	24	II	CLL	alloHSCT	Lungs	<i>P. jirovecii</i>	†
	25	II	Lymphoma	-	Lungs	<i>P. jirovecii</i>	Survival

During the literature review conducted throughout the work with this thesis, it was found that published reports on the diagnostic accuracy of betaglucan for the diagnosis of IFD in the hematology setting show very diverse results<sup>113,138-145</sup>. This was also highlighted in a meta-analysis, where the authors conclude that one major limitation to the diagnostic studies included in the meta-analysis was an incomplete reporting on timing of betaglucan measurements in relation to the time of diagnosis of IFD<sup>146</sup>.

Due to the retrospective nature of paper I, betaglucan sampling was not entirely systematic. For the patients with IFD, we thus used the mean value of betaglucan from the samples (range 1-3) collected during a time period of  $\pm 1$  week from fulfilment of the IFD reference standard to calculate the sensitivity. For the patients who did not develop IFD during the study period we used the mean value of all collected samples to calculate the specificity. The results of paper I give an overview of the diagnostic performance of betaglucan, but the reported specificity may be overestimated considering the large amount of betaglucan samples collected during the study period.

In paper II, we had a different approach. Estimates of sensitivity were instead reported for two specific time points: at the time of diagnosis (TOD) of IFD and within two weeks from the TOD. In the calculations we thus used the betaglucan results from the samples collected at the respective time point. For the patients without IFD we used the maximum rather than the mean value of betaglucan from all samples collected during the study period to calculate the specificity. This approach gives a more informative estimate of sensitivity, but may, however, underestimate the specificity.

The results of paper I and paper II, highlight the importance to take into consideration the study design and the sources of variation in diagnostic studies when interpreting results and determining their applicability to different clinical scenarios<sup>137,147</sup>.

The estimates of sensitivity and specificity reported in **paper II** are shown below.

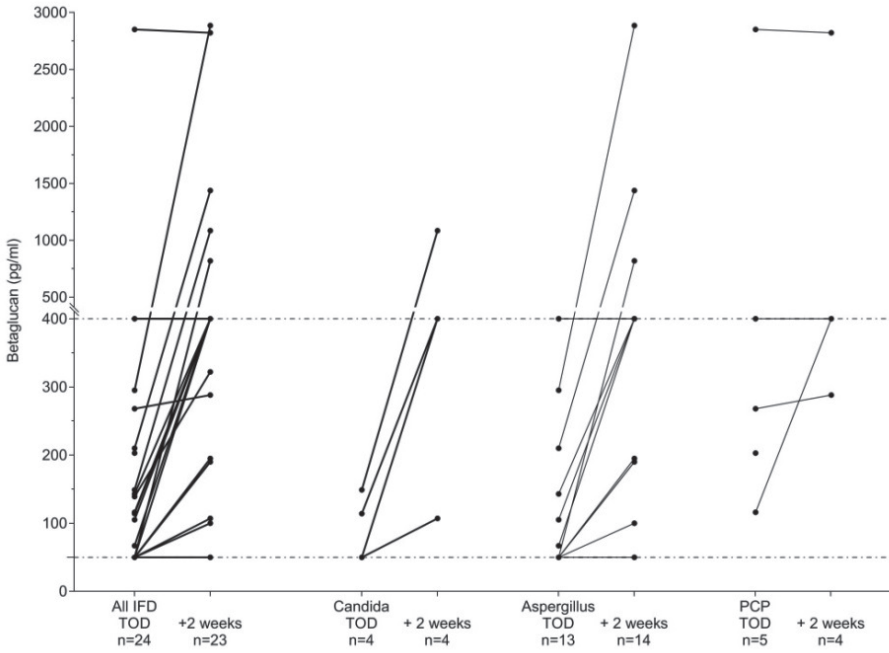
*Table 3. Estimates of diagnostic accuracy of betaglucan for the diagnosis of IFD in the prospective hematology cohort (paper II).*

Cut-off level betaglucan (pg/ml)	Sensitivity % (95% CI)		Specificity % (95% CI)
	TOD	+ 2wk	
one >80	69 (42-87)	92 (65-99)	41 (32-50)
one >160	46 (23-70)	83 (55-97)	70 (61-77)
one >400	15 (3-42)	58 (32-81)	88 (80-92)
two >80	69 (42-87)	92 (65-99)	80 (72-87)
two >160	46 (23-70)	83 (55-97)	87 (79-92)
two >400	15 (3-42)	58 (32-81)	94 (88-97)

The sensitivity of betaglucan collected at the time of diagnosis of IFD was low; however, the sensitivity was significantly higher when the test was collected 2 weeks later. Similarly, in an interesting study by Angebault *et al.* including 143 patients with IFD, it was found that only 52 and 62% of patients with invasive aspergillosis and candidemia, respectively, tested positive for betaglucan at the time of diagnosis<sup>148</sup>. Similar results showing a low sensitivity for betaglucan early in the time course of fungal disease in patients with hematologic disorders were also shown by other authors<sup>149-151</sup>.

For presentation in this thesis, the results of paper I were reassessed by the same approach used in paper II. Figure 16 shows the betaglucan values for all patients with IFD in **papers I and II** at the time of diagnosis and 2 weeks later. For the majority of patients, there is a high percentage increase in betaglucan levels between the first and second sampling episode. The results do not account for the start of antifungal treatment (which for the majority of patients occurred at some time point between the time of diagnosis and the following two weeks). However, studies investigating the betaglucan response after initiation of anti-fungal treatment in patients with IFD showed that betaglucan levels decreased slowly during the process of clinical cure<sup>149,152,153</sup>, or remained unchanged during the time of follow-up<sup>154-156</sup>. It thus seems unlikely that anti-fungal treatment would have affected the betaglucan levels during this time period of two weeks.

Figure 16. Betaglucan levels at the time of diagnosis and two weeks later, respectively. The figure includes patients with IFD from paper I and paper II.



### 4.1.3 Predictive values

As previously stated, the predictive values of a diagnostic test are dependent upon the pre-test probability of the disease in the sampled population. Table 4 shows the predictive values of betaglucan for the diagnosis of IFD for different sampling scenarios with different pre-test probabilities in the prospective hematology cohort (**paper II**).

Cut-off level betaglucan (pg/ml)	Screening high-risk patients once to twice weekly (pre-test probability 12%) n=125		Testing patients with clinically suspected IFD (pre-test probability 35%) n=37	
	PPV	NPV	PPV	NPV
one >80	0.14	0.91	0.50	0.79
one >160	0.17	0.90	0.86	0.77
one >400	0.15	0.88	1.0	0.69
two >80	0.32	0.95	0.75	0.84
two >160	0.33	0.92	0.86	0.77
two >400	0.25	0.89	1.0	0.69

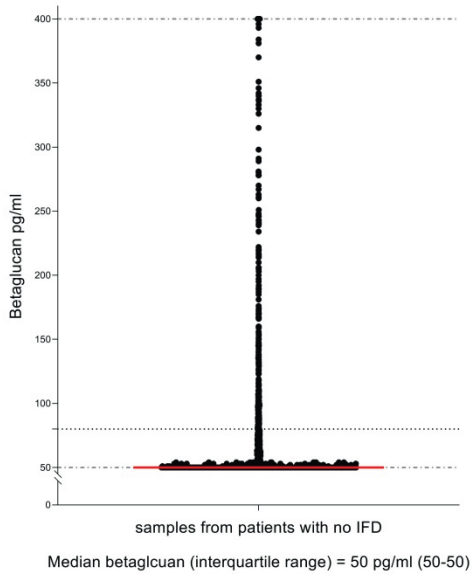
*Table 4. Predictive values of a positive betaglucan test for the diagnosis of IFD in high-risk hematology patients. The screening scenario includes all study patients that were tested for betaglucan once to twice weekly during the period of high risk for IFD. For the patients that did not develop IFD, the maximum betaglucan value during the test period was used for cross tabulation calculations. The scenario of testing for betaglucan upon clinical suspicion of IFD includes 37 patients with clinically suspected IFD. For the patients without IFD, the betaglucan value at the time point of clinical suspicion was used for cross tabulation calculations. For both scenarios, the value of the fungal marker at the time of diagnosis (TOD) in 13 patients with probable or proven IFD was used.*

In analogy with previous studies<sup>138,145</sup>, the positive predictive value of a positive betaglucan test when using betaglucan as a screening marker for IFD was low. The negative predictive value was high; however, this may not be surprising considering the relatively low prevalence of IFD and the low pre-test probability with this diagnostic approach. On the other hand, and in accordance with other studies<sup>142,157</sup>, the positive predictive value is higher when testing patients upon clinical suspicion of IFD.

#### 4.1.4 Elevated betaglucan levels in patients without IFD

Several other studies have shown that betaglucan may be elevated in patients without IFD. This was confirmed in papers I and II. Figure 17 shows the betaglucan results from 193 patients without signs of IFD included in **papers I and II**.

Figure 17. Betaglucon levels in 3088 samples from 193 patients without IFD from the hematology cohorts (paper I and II).



In **paper I**, we investigated possible sources of elevated betaglucon in patients without IFD. In these patients, three factors were found to be associated with positive betaglucon levels: admission to ICU; previous administration of plasma, coagulations factors or albumin; and ongoing treatment with pegylated asparaginase (peg-asparaginase). Our results confirmed the results of previous studies reporting that blood components may be contaminated with betaglucon due to filtration with cellulose filters during the manufacturing process<sup>158,159</sup>. Additionally, a recent study confirmed our results by showing that betaglucon concentrations in blood components may predict false positive post-transfusion results<sup>160</sup>. However, to the best of our knowledge, treatment with peg-asparaginase has not previously been reported as a cause of elevated betaglucon levels. We hypothesized that triglyceridemia, a known side effect of peg-asparaginase, may be the cause of elevated betaglucon levels in these patients. In **paper II**, we tested this hypothesis and did find a correlation between betaglucon and triglycerides in serum ( $r=0.7$ ). This has not been reported previously.

In paper I, we additionally found a higher mean betaglucon level in patients without IFD receiving intravenous immunoglobulins than in patients not receiving immunoglobulins although the difference did not reach statistical

significance. This association has also been shown in other studies<sup>159,161</sup>; and in a recent paper, elevated betaglucan levels were found in serum up to three weeks after intravenous immunoglobulin administration<sup>162</sup>. We did not find any association between positive betaglucan levels and administration of intravenous antibiotics. Although some older reports have shown that some batches of antibiotics for intravenous administration may contain betaglucan<sup>163,164</sup>, this has not been shown to give elevated betaglucan levels in patients receiving such products<sup>163,165-167</sup>. In analogy with other reports, we did not find any correlation between elevated betaglucan and concomitant bacteremia or dialysis<sup>168,169</sup>. The possibility that fungal translocation may give rise to elevated betaglucan levels in serum in patients with fungal mucosal colonization and chemotherapy-induced mucositis has been previously investigated, but a recent study did not find any correlation between biomarkers of intestinal mucositis and betaglucan levels in serum<sup>170</sup>. **In paper II**, we evaluated the association between fungal mucosal colonization and betaglucan levels in serum among 67 patients who performed serial fungal surveillance cultures in addition to betaglucan sampling. No correlation was found between colonization index (*Section 4.2*) and betaglucan levels in serum ( $r=-0.1$ , unpublished data).

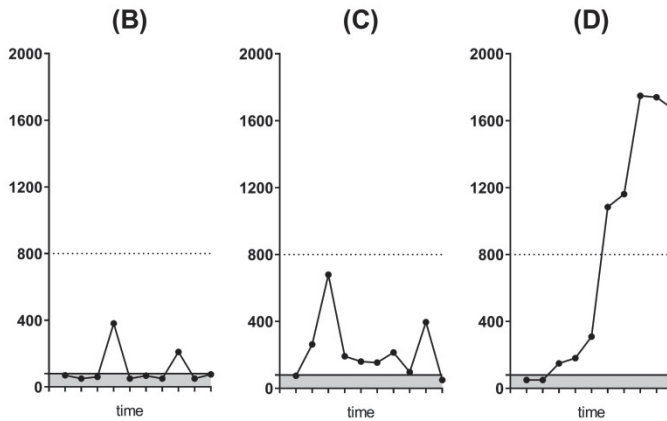
In addition to considering the above mentioned factors as possible reasons for elevated betaglucan levels in the absence of IFD, three other strategies that may be used to minimize the clinical problem with “false” positive betaglucan levels were reported in **paper II**.

First, the use of two consecutive positive test results as the determinant of a positive betaglucan result increases specificity and positive predictive values substantially (shown above in tables 3 and 4). Similar results have been shown in other studies<sup>138,142,145</sup>.

Second, titration of serum samples for quantification of betaglucan levels above the maximum level of detection aids in the interpretation of positive test results. When titrating samples from patients that initially had a betaglucan level >400 pg/ml, it was found that contrary to the patients with IFD, all patients without IFD had betaglucan levels lower than 800 pg/ml. Thus, betaglucan levels >800 pg/ml are highly indicative of IFD while samples <800 pg/ml in patients with no clinical suspicion of IFD may originate from other sources than fungal disease.

Last, evaluating the pattern of betaglucan dynamics showed that 86% of the patients without IFD but with at least one positive betaglucan sample during the sampling period had a pattern of only one isolated positive sample (B) or

fluctuating levels of positive samples (C), in contrast to the patients with IFD who had consecutively positive samples with steadily increasing levels (D):



#### 4.1.5 Further methodological considerations

Some further methodological considerations to **papers I and II** deserve to be mentioned.

A limitation to papers I and II is the limited number of patients with IFD (13 in each study). This affects the robustness of the reported estimates of sensitivity and positive predictive value, as can be seen by the large confidence intervals.

Another factor to be considered is the presentation of ‘IFD’ as one disease entity. In light of the diversity of the spectrum of invasive fungal disease described in the introduction, it would be desirable to report results of diagnostic accuracy for the different types of IFD separately. However; considering the low prevalence of IFD, longitudinal cohort studies enabling for stratification into different types of IFD would require multicenter studies with very large sample sizes which may not be feasible.

Finally, in the case of IFD there may not be a clear threshold to distinguish between the presence and absence of disease. The reference standard thus includes levels of probability of disease, as previously described in section 3.2.1. The decision to include or exclude levels of low probability of disease (*i.e.* ‘possible IFD’) is not straightforward, and regardless of which approach is chosen, misclassification bias may occur that may overestimate or underestimate the results of diagnostic accuracy<sup>135,171</sup>. In papers I and II we



chose to exclude the group classified as “possible IFD”. This results in a stricter and perhaps more clinically relevant definition of the target condition, which may be an advantage when determining the diagnostic accuracy of a test. On the other hand, this may also lead to an overestimation of the sensitivity of the test.

#### 4.1.6 HIV-cohort (Paper III)

**Paper III** was a case-control study aimed to assess the utility of serum based microbiological analyses, including *Pneumocystis* PCR and betaglucan, for the diagnosis of *Pneumocystis* pneumonia in HIV-infected patients. HIV-infected patients without PCP and blood donors were included as negative controls.

We found that 96% of the 26 patients with PCP had positive betaglucan levels (>80 pg/ml). This yielded a high sensitivity of betaglucan for the diagnosis of PCP in this cohort. Similarly, other studies<sup>172,173</sup>, including two meta-analyses<sup>174,175</sup>, have shown a high diagnostic accuracy of the betaglucan test in serum for the diagnosis of PCP in HIV-infected and non HIV-infected patients. As previously discussed, the timing of betaglucan sampling is important to consider when interpreting the results of sensitivity and negative predictive values. In our study, the betaglucan samples were collected at the time of PCP diagnosis and prior to the start of PCP treatment. The median duration of respiratory symptoms prior to PCP diagnosis was 28 days. It is known that HIV-infected patients with PCP typically have a slow progression of symptoms<sup>42,49</sup> which may result in a delay to seek health care. In many studies evaluating betaglucan as a diagnostic marker for PCP in HIV-infected patients, betaglucan sampling is thus performed after several weeks of symptoms, which may contribute to the reported high sensitivity.

In paper III, none of the blood donors had detectable betaglucan levels in serum while 14% of the HIV-infected negative control subjects had positive betaglucan levels. This yielded a moderate specificity of betaglucan for the diagnosis of PCP in the HIV-infected cohort. However, further investigations of the HIV-infected negative controls revealed that some of these patients may in fact have had a mild and self-limiting *Pneumocystis* infection, why the specificity may have been underestimated (further discussed in *Section 4.3*).

### 4.1.7 Lung transplanted cohort (Paper IV)

In paper IV, betaglucan levels in serum and BAL fluid were evaluated in lung transplant recipients with fungal tracheobronchitis.

In total, 37 and 17 patients with *Candida* and *Aspergillus* tracheobronchitis, respectively, were identified in this prospective cohort study. The median betaglucan level in patients with *Candida* or *Aspergillus* tracheobronchitis was less than 80 pg/ml in serum for both groups but 228 and 129 pg/ml in BAL fluid, respectively. There was a statistically significant difference in median betaglucan levels in BAL fluid in patients with fungal tracheobronchitis compared to patients with no Fungi in BAL fluid.

ROC curve analyses for the betaglucan test in BAL fluid in patients with *Candida* and *Aspergillus*, respectively, were performed for the following groups:

- A. patients with fungi isolated from BAL-fluid compared to patients with no fungi
- B. patients with tracheobronchitis compared to patients with no fungi
- C. patients with tracheobronchitis compared to patients with colonization

The highest area under the ROC-curve (0.8) was obtained for the *Aspergillus* group, scenario B.

For scenario C, the area under the ROC-curve was 0.58 for both the *Candida* and *Aspergillus* groups.

Considering the high frequency of fungal isolation from BAL fluid following lung transplantation<sup>176,177</sup>, a test that could discriminate between airway invasive disease and colonization would be useful. Our results, supported by other reports<sup>178,179</sup>, show that the betaglucan test in serum has no role in the diagnosis of fungal tracheobronchitis caused by *Candida* or *Aspergillus* in lung transplant recipients, which may be explained by a low degree of tissue invasiveness and a low fungal burden in blood in patients with fungal tracheobronchitis. The betaglucan test performed on BAL fluid showed a somewhat higher diagnostic accuracy when comparing patients with tracheobronchitis and patients with no fungi in BAL fluid; however, it was not able to discriminate patients with fungal tracheobronchitis from patients

with fungal colonization. In analogy, a meta-analysis including six studies evaluating the diagnostic value of betaglucan in BAL-fluid for the diagnosis of invasive fungal lung disease showed heterogeneous results with a low pooled sensitivity and specificity<sup>180</sup>.

An important limitation to this study is the use of an unestablished reference standard to define cases of probable *Candida* tracheobronchitis. However, the definition of proven *Candida* tracheobronchitis would require histopathological evidence of fungal elements in biopsy specimen<sup>112</sup>, and studies based on only proven cases of tracheobronchitis may not be easily feasible.

## 4.2 Diagnostic accuracy of a combination of fungal markers (Paper II)

The aim of **paper II** was to prospectively evaluate the utility of a screening method using a combination of fungal markers in high-risk hematology patients.

### Fungal surveillance cultures

In addition to the collection of serum and urine samples for the analysis of betaglucan, galactomannan, bm-gliotoxin and DA/LA ratio, fungal surveillance cultures were performed once weekly at one of the study sites (Gothenburg). The cultures were collected from feces, urine, the oral cavity and catheter insertion sites. The colonization index<sup>181</sup> (number of colonized sites/number of cultured sites) at each culture episode was calculated.

A total of 67 patients had serial fungal surveillance cultures performed (596 culture episodes), out of which 44 (66%) had at least one episode with positive fungal cultures (colonization index>0). Among all patients with at least one episode of fungal colonization, 73% were colonized by *C albicans*, 18% by *C glabrata* and 16% by *C parapsilosis*. Some patients were colonized by more than one *Candida* species. *C krusei* was not isolated from any of the surveillance culture specimens (unpublished data). This reflects the distribution of invasive *Candida* blood isolates reported in a nationwide Swedish study where *C. albicans* was the most commonly isolated species, followed by *C. glabrata* and *C. parapsilosis*<sup>5</sup>.

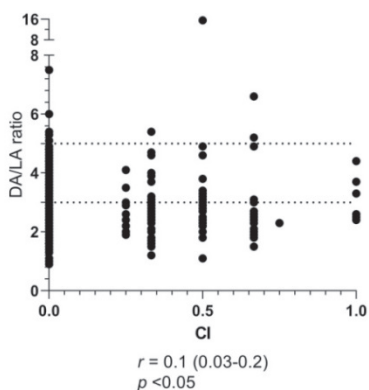
## Galactomannan

Seven patients had proven or probable invasive aspergillosis (IA). Similar to betaglucan, the sensitivity of galactomannan for the diagnosis of IA was very low when the sample was collected at the time of diagnosis of IA (33%), but in contrast to betaglucan, the sensitivity remained low when the sample was collected 2 weeks later (43%). A meta-analysis from 2015 on the diagnostic accuracy of galactomannan for the diagnosis of IA report higher sensitivities<sup>182</sup>. As previously mentioned, the issue of timing of blood sampling in relation to the time of diagnosis is essential when interpreting the results of sensitivity. However, the timing of galactomannan sampling was not included as one of the sources of heterogeneity investigated in the meta-analysis due to the fact that only 19% of the studies included in the meta-analysis provided with this information<sup>182</sup>. This may be a factor influencing the higher reported sensitivity in previous studies. Nevertheless, important to note is that the low number of patients with IA included in paper II does not allow for any firm conclusions to be drawn as to the estimate of sensitivity of galactomannan.

On the other hand, the specificity of galactomannan was >90 at both evaluated cut-off levels, and a combination of a betaglucan test >80 pg/ml and a galactomannan test >0.5 ODI yielded a very high specificity (99%) suggesting that this combination of test results is highly indicative of IA.

## DA/LA ratio

Among all collected urine samples, 80% were negative for DA/LA ratio and 17% indeterminate. There was no evidence that *Candida* colonization, expressed by the *Candida* colonization index, had any impact on the results of urinary DA/LA ratio:



*The figure shows the urinary DA/LA ratios and colonization index in 61 patients with DA-LA ratio and fungal surveillance cultures performed on the same day (345 measurements). No correlation ( $r=0.1$ ) was found between the colonization index and the urinary DA/LA ratio, although the fact that there were repeated measurements in patients does not allow for firm conclusions to be drawn.*

DA/LA ratio in urine has previously been launched as a useful marker to aid in the diagnosis of invasive candidiasis in hematologic patients, in particular *C. albicans* infections, but also infections caused by other DA-producing *Candida* spp., such as *C. parapsilosis* and *C. tropicalis*<sup>119,130,183</sup>. During the study period of 18 months in paper II, there was only one case of invasive candidiasis which was due to *C. krusei* known not to produce D-arabinitol<sup>184</sup> why the urine DA/LA test had limited utility as a screening marker for IFD in this group of patients. This reflects the current epidemiology of IFD in hematologic patients with decreasing incidence of invasive *Candida* infections due to the extensive usage of fluconazole prophylaxis<sup>67,185</sup>.

### **Bis(methyl)gliotoxin**

In the beginning of the study, a first batch of bm-gliotoxin analysis was done on 97 frozen serum aliquots from 25 study patients with clinically suspected IFD, out of which 15 patients fulfilled the EORTC criteria for invasive aspergillosis (proven n=1, probable n=4 and possible n=10). All samples were negative for bm-gliotoxin, why additional analysis on the remaining patient sera was not performed.

In a report published by Domingo *et al.*, bm-gliotoxin was detected in the sera of 10 out of 16 patients with probable or possible IA<sup>123</sup>. A later clinical study by the same research group on the diagnostic performance of bm-gliotoxin among patients at risk of IA showed a sensitivity and specificity of 62 and 93%, respectively. Despite using similar methodology<sup>123</sup>, none of the serum samples from 14 patients with proven, probable or possible IA in our study were bm-gliotoxin positive. Our inability to reproduce previously published results regarding detection of bm-gliotoxin in serum from patients with IA (including one patient with autopsy verified infection) evokes the necessity for further studies proving its applicability as a diagnostic marker of IA.

## **4.3 *Pneumocystis* PCR in serum in HIV-infected patients (Paper III)**

In paper II, we found detectable *Pneumocystis* DNA by PCR in serum samples from two patients with *Pneumocystis* pneumonia (unpublished data). This led us to design paper III, a diagnostic case-control study intended to make a first assessment of the diagnostic potential of serum based microbiological analyses with *Pneumocystis* PCR and the betaglucan test for the diagnosis of *Pneumocystis* pneumonia in HIV-infected patients.

Surprisingly, we found that all 26 patients with PCP included in the study had *Pneumocystis* DNA in serum detected by PCR, yielding a sensitivity of 100% for this serum based PCR-assay. This was a significantly higher sensitivity compared to previous reports.

Older publications evaluating *Pneumocystis* PCR on serum samples for the diagnosis of PCP showed a very limited diagnostic potential<sup>97,186-190</sup>, which in part may be explained by the use of older PCR methodology. Although more recent studies based on real-time PCR methodology are scarce, some reports have been published in the last six years. In two studies, results of *Pneumocystis jirovecii* (*Pj*) PCR in serum samples were presented in patients with mixed types of underlying immunosuppressive disorders<sup>191</sup> and in patients with HIV<sup>192</sup>. Both of these studies showed a low sensitivity for *Pj* PCR in serum. However, in these studies, the diagnostic accuracy of serum PCR was evaluated against microbiological evidence of *Pneumocystis* in the lower respiratory tract, but no clinical evaluation of the patients was included to differentiate patients with PCP from patients with *Pj* colonization. Furthermore, in one of these studies more than half of included patients had started empirical PCP treatment before blood sampling<sup>192</sup>. Additionally, in a study published in 2018, a 25% positivity rate for *Pj* PCR in plasma samples was found among 80 HIV-infected patients diagnosed with PCP according to clinical routine. However, no pre-defined reference standard for PCP was reported<sup>193</sup>.

In contrast to previous studies, the results of our study were based on a reference standard for PCP including previously defined clinical criteria as recommended by the Standards for Reporting Diagnostic Accuracy (STARD)<sup>194</sup>. This may be one factor explaining the higher sensitivity found in our study. Furthermore, differences in laboratory methodology including protocols for DNA-extraction, PCR-assays and choice of blood fraction specimens used for analysis may also account for differences across the studies. Nevertheless, the limited number of patients in our study is a factor that needs to be considered when interpreting the results. Also, the fact that the study had a case control design may also have influenced the estimates of diagnostic accuracy favourably.

In paper III, all blood donors had negative *Pneumocystis* PCR results in serum while six out of 21 HIV-infected negative control patients were positive. This yielded a specificity of 71% of this *Pneumocystis* PCR assay on serum samples for the diagnosis of PCP in HIV-infected patients.

The finding of detectable *Pneumocystis* DNA in six HIV-infected patients without PCP was target for a more in depth analysis. We found that 5/6 PCR-positive negative control patients had respiratory symptoms and at least two had some radiological abnormality. Although none of the controls had been diagnosed with nor received treatment for PCP, we cannot rule out that some of the PCR-positive controls may in fact have had a self-limiting and undiagnosed *Pneumocystis* infection, which may have underestimated the specificity of the serum PCR assay in our study. Another hypothetical explanation to the finding of circulating *Pj* DNA in HIV-infected patients without a defined PCP may be DNA derived from *Pneumocystis* organisms colonizing the respiratory tract. However, the retrospective design of this study did not allow for a complete investigation of the six PCR-positive control patients, which is a limitation to this study.

The finding of a lower concentration of *Pj* DNA (expressed by a higher median Ct value) in serum samples from the PCR-positive controls compared to the patients with PCP led us to investigate whether the use of a specific Ct-value as cut-off for a positive test-result may result in a higher diagnostic accuracy. This proved to be true and when applying a Ct value of 34 as cut-off for a positive test result, the specificity increased from 71% to 90% without a significant loss of sensitivity. This suggests that HIV-infected patients without manifest PCP may have a low concentration of circulating *Pj* DNA in the blood.

Some limitations to this study are a limited number of included patients and a retrospective case control design. Although there are reports stating that diagnostic case-control studies may overestimate the results of diagnostic accuracy<sup>135</sup>, case-control designs may be very well suited for a first assessment of diagnostic accuracy of a test<sup>195</sup>. This study was aimed at assessing the clinical relevance of the *Pj* PCR analysis in serum, and prospective, longitudinal studies are now warranted for further evaluation of our findings.

## **4.4 Aid to researchers conducting diagnostic accuracy studies**

During the work with this thesis, some difficulties in conducting diagnostic studies became evident; not least in the field of IFD, where there is a large number of clinical variables that can potentially affect the accuracy of diagnostic tests. Fortunately, such difficulties have also been highlighted in several reports during the last years.

In an interesting publication, Whiting *et al.* present a systematic review listing sources of bias and variation in diagnostic test accuracy studies and describe the effects such variations may have on the results of such studies. The authors emphasize that although there may be several potential sources of bias to the results of diagnostic accuracy, other factors should not be interpreted as bias but rather as sources of variations that may result in true differences in diagnostic accuracy. When evaluating diagnostic accuracy studies, it is thus essential to consider such sources of variation; and rather than generalizing results across studies, the results should be put in relation to their relevance for each clinical scenario<sup>135</sup>.

As an aid to researchers conducting systematic reviews of diagnostic accuracy studies, the ‘QUADAS-2 tool’ was developed by the same authors. This tool includes four issues that should be investigated in the analysis of primary diagnostic accuracy studies, which are; patient selection, index test, reference standard, and flow and timing<sup>196</sup>.

The importance of including a detailed and informative methodology in primary diagnostic accuracy reports is thus obvious. Nevertheless, many reports lack essential information, impeding critical evaluation of the results<sup>194</sup>. In an attempt to improve the quality of reporting of diagnostic studies, the updated Standards for Reporting Diagnostic Accuracy (STARD) statement were published in 2015. In this report, the authors provide the researchers with a list of ‘essential items’ that should be reported in all studies dealing with the evaluation of diagnostic tests in order to facilitate the interpretation of results<sup>194</sup>.

## **4.5 Clinical impact of fungal tracheobronchitis (Paper IV)**

Finally, in paper IV, we evaluated the clinical impact of fungal colonization and tracheobronchitis on the development of BOS and on all-cause mortality. A total of 126 Swedish lung transplant recipients were followed prospectively during the first year after transplantation. The development of BOS was assessed during a maximum follow-up time of 6.4 years. The distribution of patients classified as colonized and with tracheobronchitis, respectively, during the first year after transplantation is shown in Table 5. Sixty patients (48%) developed BOS and 42 patients (33%) died during the total follow-up.



Table 5. Classification of patients with fungal isolation in BAL fluid

Patients, n	126
Candida in BAL during the first post-operative year, n of patients (%)	54 (43)
Colonization	17
Tracheobronchitis	37
Aspergillus in BAL during the first post-operative year, n of patients (%)	29 (23)
Colonization	12
Tracheobronchitis	17
BOS, n (%)	60 (48)
Deaths (all-cause), n (%)	42 (33)

The cumulative one-year incidences were as follows:

	<b>One-year cumulative incidence (%)</b>
<i>Candida</i> in BAL fluid	43
<i>Candida</i> tracheobronchitis	30
<i>Aspergillus</i> in BAL fluid	24
<i>Aspergillus</i> tracheobronchitis	14

The one-year incidence of fungal presence in the lower respiratory tract of Swedish lung transplant recipients was high. Previous studies assessing the presence of fungal colonization after lung transplantation have focused primarily on colonization by *Aspergillus* species, and incidence rates of approximately 30% have been reported<sup>177</sup>, similar to the one year incidence of *Aspergillus* colonization found in our study. To the best of our knowledge there are no previous studies reporting the incidence of fungal tracheobronchitis after lung transplantation, including cases of *Candida* tracheobronchitis. Compared to the one-year incidences of invasive pulmonary *Aspergillus* infections (including tracheobronchitis) in lung transplant recipients of 6-11% reported in other studies<sup>88,197,198</sup>, the incidence of *Aspergillus* tracheobronchitis found in our study was high. Some factors that may explain differences in the reported incidence of fungal infections are local routines for anti-fungal prophylaxis and variations in the definitions used to classify the cases of fungal infections.

Although the clinical relevance of *Candida* presence in BAL fluid may be considered very low in other patient groups, *Candida* tracheobronchitis is a recognized complication in lung transplant recipients in the early post-operative period<sup>90</sup>. It is generally accepted that colonization or infection by different microorganisms is associated with the development of chronic

allograft loss in terms of bronchiolitis obliterans syndrome (BOS) after lung transplantation<sup>199</sup>. Weight *et al* reported that the presence of *Aspergillus* in the lower respiratory tract in lung transplant recipients significantly increased the risk for BOS<sup>200</sup>, but to the best of our knowledge, no studies have previously assessed the impact of *Candida* colonization or tracheobronchitis on the development of BOS.

The following “fungal status” predictors, for *Candida* and *Aspergillus* separately, were analyzed by Cox multivariate regression to assess the impact on the *development of BOS*. The variables ‘cyclosporine’ and ‘acute rejection grade A2 or higher’ showed a significant impact on the development of BOS in a previous univariate analysis and were thus included in the multivariate model as covariates.

“Fungal status” predictor	<i>Candida</i>		<i>Aspergillus</i>	
	HR	<i>p</i>	HR	<i>p</i>
Fungal colonization	0.89	0.76	0.96	0.92
Fungal tracheobronchitis	0.70	0.27	0.92	0.83
Fungal presence once	0.87	0.65	1.04	0.92
Persistent fungal presence	0.52	0.17	0.75	0.59
Fungal presence AND BAL betaglucan ≥80pg/ml	0.76	0.41	1.0	0.99
Fungal presence AND BAL betaglucan <80pg/ml	0.49	0.18	0.82	0.71

We found no association between the presence of *Candida* or *Aspergillus* in BAL fluid (for any of the predictors) and the development of BOS. Our results differ from those reported by Weight *et al*<sup>200</sup> regarding *Aspergillus* and an increased risk for BOS. The lower number of patients and the lower median follow-up time in our study may be influencing factors. Furthermore, whether or not variations in treatment routines in the different studies may have influenced the results remains unknown. The long term impact of *Candida* in BAL fluid was, to the best of our knowledge, not previously reported.

We additionally determined the impact of fungal colonization and tracheobronchitis on all-cause mortality. We found no difference in survival between the patients colonized or infected by fungi and the patients with no fungi isolated from BAL fluid. The overall 6-year survival in our

cohort was approximately 60% both among patients with and without fungal presence, which is in line with the 6-years survival of patients without fungi reported in the aforementioned studies<sup>201-203</sup>.

In summary; contrary to other studies, we found no clinical impact of *Candida* or *Aspergillus* colonization or tracheobronchitis neither on the development of BOS or on all-cause mortality. This was a somewhat surprising finding that warrants further investigation.

## 5 CONCLUSION AND FUTURE PERSPECTIVES

In the last decade, there have been significant advances in the research field of diagnostic aspects of invasive fungal disease in immunocompromised hosts, in particular regarding different non-culture diagnostic methods. Today, a MEDLINE search including various keywords and syntax forms for ‘betaglucan’, ‘mycoses’ and ‘diagnosis’ yields approximately 950 publications published during a time frame of thirty years, out of which sixty percent were published during the eight years of work with this thesis. There are still uncertainties regarding the optimal clinical use of betaglucan, yet most recent international guidelines include a recommendation to use betaglucan as a diagnostic marker for IFD<sup>26,55,177,204,205</sup>. Considering the complexity of fungal-host interactions, which most likely affects the dynamics of fungal markers in blood, the assessment of diagnostic performance of different fungal markers is challenging, and as discussed in this thesis, estimates of diagnostic accuracy should be interpreted in relation to the design of the study and to the clinical applicability. The importance of following published guidelines in order to achieve a high quality of reporting of diagnostic studies has been highlighted and this is certainly an important perspective for future studies regarding diagnosis of IFD.

The papers included in this thesis contribute with some further knowledge to the field of IFD, in particular regarding diagnostic aspects. Here follows some of the findings:

- There is a large inter-patient variation when measuring betaglucan levels in serum
- The sensitivity of betaglucan for diagnosing mixed types of IFDs in patients with hematologic malignancies and alloHSCT recipients may be low early during the course of disease. However, betaglucan levels seem to increase rapidly and if performing the betaglucan test at a later point during the course of infection (within 2 weeks), the sensitivity is significantly higher. Betaglucan may thus not be suitable for early detection but rather for confirmation of fungal disease.
- The positive predictive value of betaglucan is higher if performing the test upon clinical suspicion of IFD than if using betaglucan as a screening marker for IFD in high-risk patients in the hematology setting.

- To optimize the positive predictive value, a higher cut-off level than that recommended by the manufacturer and two consecutive positive test results to confirm the positive result should be used.
- Admission to ICU; previous administration of plasma, coagulations factors or albumin; and high triglyceride levels in serum may cause elevated betaglucan levels in patients without IFD.
- Betaglucan levels above 800 pg/ml and a pattern of consecutively positive and increasing betaglucan levels are both highly indicative of IFD in patients with hematologic disorders.

The above mentioned findings regarding the clinical applicability of betaglucan for specific clinical scenarios, is a relevant issue that deserves further in depth investigation in studies including larger patients groups and patients with other underlying disorders.

- Betaglucan testing in serum seems to have no role in the diagnosis of fungal tracheobronchitis in lung transplant recipients; however, betaglucan levels in BAL fluid may be elevated in these patients. Additional analyses are needed to clarify possible explanations to the diversity of measured betaglucan levels in BAL fluid among patients with fungal colonization and tracheobronchitis.
- The combination of a positive betaglucan and a positive galactomannan test has a high specificity for the diagnosis of invasive aspergillosis in patients with hematologic disorders.
- The use of bis(methyl)gliotoxin for the diagnosis of invasive aspergillosis in the hematology setting seems questionable.
- *Pneumocystis* PCR in serum samples may be a highly sensitive method for diagnosis of PCP in HIV-infected patients. This is promising data which will be further explored in prospective longitudinal study including different patient cohorts.
- *Candida* and *Aspergillus* is frequently isolated from BAL fluid of lung transplant recipients during the first year after transplantation. The cumulative one-year incidence of *Candida*

and *Aspergillus* tracheobronchitis in Sweden is 30 and 14%, respectively.

- Fungal colonization or tracheobronchitis seems to have little impact on all-cause mortality or on the development of BOS during 6 years follow up. This deserves further exploration, and additional analyses regarding other potential covariate predictors, such as receipt of anti-fungal treatment and different microbiologic markers are warranted.

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