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## **Risk factors and new markers of pulmonary fungal infection**

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# Chapter 1

## **GENERAL INTRODUCTION & OUTLINE OF THE THESIS**



## General Background

### Pulmonary fungal infections in the transplant recipient

After their introduction in the early 1960s, solid organ and bone marrow transplantation have now become established treatment options for a wide range of potentially fatal disorders. This advance in the field of medicine also heralded a new era in the field of prevention, diagnosis and treatment of opportunistic fungal infections. Prior to this time, these infections were encountered infrequently in patients receiving intensive chemotherapy, suffering from malnourishment or from congenital or acquired immunological disorders [1, 2]. Because of the relatively low incidence of diseases caused by these pathogens, interest in exploration of the specific fungal host-pathogen interactions was limited. This however changed along with the rising numbers of patients with a severely compromised immune system and the parallel increase in the incidence of fungal infections. It soon became recognized that this emerging class of pathogens was responsible for a high morbidity and mortality in the transplanted population [3-5].

The timing and type of fungal infection correlates with the specific immunodeficiency imposed by underlying disease or treatment modalities received by the patient [6]. *Pneumocystis pneumonia* (PCP), caused by infection with the fungus *Pneumocystis jirovecii*, may only develop under the condition of T-cell lymphocyte depletion or dysfunction. After the spread of the HIV epidemic in the 1980s, increasing attention was drawn to this infection as the PCP incidence climbed to >50% in patients presenting with AIDS [7, 8]. Although progress has been made with regard to unraveling of the biology and lifecycle of *Pneumocystis* in the past decades, a more profound understanding of the pathogenicity, transmission and epidemiology has been severely hampered by the lack of available in-vitro culture methods. With the advent of highly active antiretroviral therapy (HAART) in 1996 and the protocolized use of chemoprophylaxis, the incidence of PCP in HIV-infected patients dropped dramatically [9]. In contrast, the number of patients at risk for PCP due to solid organ or hematopoietic stem cell transplantation continues to increase. Chemoprophylaxis also proved effective in preventing PCP in solid organ transplant recipients [10, 11]. However, deficits in our understanding of risk factors and mode(s) of transmission preclude the identification of patients at high risk and settings in which outbreaks of PCP may occur [12]. Hence, PCP has remained to be a frequently considered diagnosis in transplant recipients presenting with interstitial pneumonia.

For patients subject to myeloablative chemotherapy or hematopoietic stem cell transplantation, the peak incidence of fungal infection is found during episodes of neutropenia [13]. In this setting *Aspergillus* (a filamentous fungus belonging to the family of *Trichocomaceae*) is recognized as the most important cause of severe pulmonary fungal infection [14]. After inhalation into the small airways and alveoli, infectious conidia that overcome the innate

immune defenses germinate and form hyphae. If unchallenged by the host's cellular immune response (i.e., neutrophilic granulocytes), angioinvasive hyphal growth leads to pulmonary hemorrhage and respiratory failure. Even in the era of effective antifungal drugs, a large proportion of severely immunocompromised patients diagnosed with invasive aspergillosis experience treatment failure, which carries a mortality of approximately 30% [15]. The reversal of immune suppression, e.g., by engraftment of hematopoietic stem cells and return in the blood of neutrophilic granulocytes, has remained the best predictor of control of invasive aspergillosis and recovery of the patient [16].

## ***Pneumocystis pneumonia***

### *Pneumocystis*

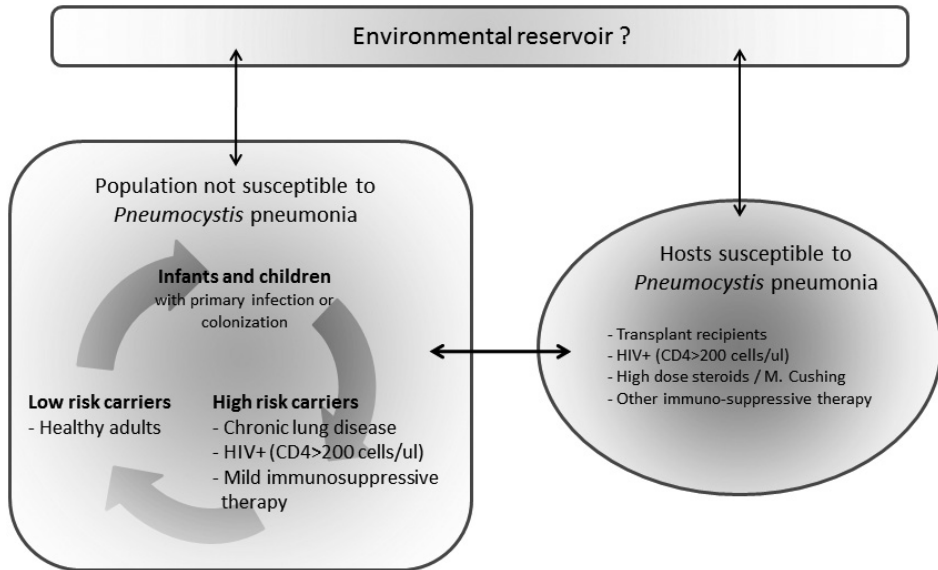
The *Pneumocystis* genus consists of multiple individual species that each require a specific mammalian host [17]. Accompanying this selective infectivity, morphological and biological differences between separate species have been demonstrated. At the electron microscopic level, individual *Pneumocystis* species show distinctive morphology of the filopodia as well as different densities of the membrane-limited cytoplasmic granules [18, 19]. The species that causes pneumonia in humans has been renamed *Pneumocystis jirovecii*, but was previously known as *Pneumocystis carinii* or *Pneumocystis carinii* f. sp. *hominis* [20, 21].

For a long time *Pneumocystis* was considered a protozoa by the majority of the medical community involved in *Pneumocystis* research and treatment of PCP. This was due to morphological characteristics, the lack of growth in various fungal culture media and the apparent inability to cure patients with the classical anti-fungal agents Amphotericin B and Ketoconazole. In contrast, other drugs like Trimethoprim-Sulfamethoxazole and Pentamidine, commonly used in the treatment of protozoan infections, were used with marked success in the treatment and prevention of PCP. These drugs inhibit metabolic pathways partially common to protozoa and fungi. The lack of responsiveness to polyenes and triazoles is now known to be due to the lack of ergosterol in the cell membrane of *Pneumocystis*, which is replaced by cholesterol [22]. At present, *Pneumocystis* is classified as a fungal organism on the basis of DNA analyses [23].

### *Transmission and Susceptibility*

The primary source of *Pneumocystis* causing infection in humans has been heavily debated, and it was thought for a long time that it had an environmental origin [24]. In animal models it was convincingly demonstrated that host-to-host transmission could occur via the airborne route [25]. In humans however, observations point to the possibility of either endogenous reactivation or an exogenous source being the major cause of infection [26]. Studies performing serology for *Pneumocystis* showed that the fungus is contacted already early in life, with over 50% of 8-month-old and 85-100% of 2-year-old children having specific anti-*Pneumocystis* antibodies [27, 28]. In recent years over 20 studies reported the asymptomatic carriage of

**Figure 1.** Potential model and routes of transmission of *Pneumocystis*, modified from Peterson and Cushion, reference [34]. Arrows indicate transmission routes. \*Mild infection with flu-like symptoms may occur during primary infection in, for *Pneumocystis* naive, children.



*P. jirovecii* in all kinds of human subpopulations consisting of healthy or immunocompromised adults [29-32]. Together with recent information derived from genotypic studies of *Pneumocystis* organisms isolated during PCP outbreaks in hospitals this strongly indicates that interhuman transmission is the major route of infection. At present, the predominant hypothesis comprises that groups at risk for carriage provide the main species-specific reservoir of *P. jirovecii* and that environmental reservoirs may contemporary co-exist through exhalation of Pneumocysts in the air [33]. Host at risk then become infected by inhalation and develop PCP (figure 1).

The CD4+ T-cell count is the best predictor of risk for PCP in HIV-positive patients. Recent work further suggest additional but independent influence of HIV replication itself [35]. Though, the CD4+ T-cell count is more difficult to use as a reliable cut-off for the need of PCP chemoprophylaxis in HIV-negative immunocompromised populations [36]. Only a few, small studies explored the clinical risk factors for PCP in transplant recipients. In general, superimposed degrees of iatrogenic immune suppression due to intensive maintenance immunosuppression, treatment for graft rejection and concurrent CMV infection are probably predictive for development of PCP in this population [37-39].

#### *Immune response to Pneumocystis*

In the majority of patients the primary immune impairment is T-lymphocyte depletion or dysfunction. The central and crucial role of CD4+ T-cells in the defense against *Pneumocystis* has been appreciated ever since the association with advanced HIV infection became clear.

Additional research showed that colonization was associated with low CD4+ T-cell counts and CD4+/CD8+ T-cell ratio's <1 [40, 41]. Besides the established role of CD4+ T-cells, uncertainty exist about the role of CD8+ T-cell subsets [42]. Nonetheless, the actions of T-lymphocytes do not stand alone but mediate a complex immune response that involves components of the innate, humoral and cellular immunity [43, 44]. The inflammatory response to *P. jirovecii* not only promotes the essential clearance of this microorganism from the lungs but also causes the collateral damage to the lung tissue. This results in decreased efficacy of gas exchange and associated symptoms of respiratory distress [45].

Pneumocysts are initially recognized by alveolar macrophages through Dectin-1 and other receptors, which interact with glycoprotein moieties ( $\beta$ -D-glucan, glycoprotein A and the *Pneumocystis* major surface antigen) [46, 47]. This process is enhanced by binding of fibronectin and vitronectin to the *Pneumocystis* cell wall and counteracted by down regulation of both surfactant protein A and B and up-regulation of surfactant protein D [48]. The relevance of these components that operate in the initial encounter with *Pneumocystis* has been confirmed in animal and in-vitro models. For example, Toll-like receptor 2 or 4 were found to be important mediators of the immune response in animal models with induced *P. murina* pneumonia [49, 50]. However, in another study, no direct effect on the phagocytosis capacity of alveolar macrophages was noted [51]. This is likely due to redundancy of the signalling pathways to NF- $\kappa$ B activation, since a single deficiency of one of the recognition 'molecules' did not exclude an effective activation of this pathway [52-54]. NF- $\kappa$ B propagates the release of pro-inflammatory cytokines, IL-1, IL-8 and TNF. The latter molecule stimulates alveolar epithelial cells (Pneumocytes type I) to increase cytokine production and recruits monocytes, CD8+ cytotoxic lymphocytes as well as neutrophils into the alveolar space. Among the other cytokines investigated, IFN- $\gamma$  in particular is noteworthy because of its ability to induce macrophage activation and to exert inflammatory effects by T-cells. Neutralization of IFN- $\gamma$  reduced survival in a rat model for *Pneumocystis* pneumonia [55]. Because *P. jirovecii* cannot be cultured in vitro, the study of the pathogenesis of PCP largely has largely been restricted to animal models [56, 57].

#### *Diagnosis of Pneumocystis pneumonia*

The diagnosis of PCP is currently based on direct microscopy using silver, giemsa and immunofluorescent staining and real-time PCR performed on broncho-alveolar lavage samples [58]. Several issues complicate these techniques. Microscopical methods have limited sensitivity, require well trained and experienced personnel and involve time demanding procedures. On the other hand, real time PCR methods yield high sensitivity and can be implemented as a rapid routine diagnostic test, but might lack the required specificity since it may also detect *P. jirovecii* in patients who are colonized with *P. jirovecii* but do not suffer from PCP [30, 59-62]. A *P. jirovecii* antibody test was developed but failed to yield sufficient diagnostic power [63]. This is probably caused by the pre-existing antibody response to *P. jirovecii* [28, 64].

When sampling of the lower airways cannot be performed for clinical reasons, the diagnosis of PCP relies solely on clinical signs and chest imaging. In such situations the need for a sensitive and specific, non-invasive test for PCP becomes particularly urgent. A number of serum markers were recently studied for their ability to discriminate between PCP and other pulmonary conditions in patients infected with HIV [65-67]. It remains questionable whether advances made in prevention strategies and diagnostic markers for PCP in HIV-positive patients can be extrapolated to the HIV-negative population at risk. HIV-related PCP and non-HIV related PCP are known to be different in terms of clinical characteristics [68, 69]. Autopsy studies demonstrate that higher loads of *P. jirovecii* are present in the lungs of patients with HIV as compared to patients with PCP due to other underlying disorders [70, 71]. Interestingly, the inflammatory reaction evoked by *Pneumocystis* is relatively more severe in HIV-negative immunocompromised individuals with PCP [69, 72]. This probably reflects a less impaired immunity as compared to end stage HIV patients. Prospective studies that address the clinical utility of serum markers for diagnosing PCP in solid organ transplant recipients and in patients with other causes of immunodeficiency are needed [73].

## Invasive Aspergillosis

### *Aspergillus* and Invasive aspergillosis

The term 'invasive aspergillosis' refers to tissue invasion by the filamentous fungus *Aspergillus*, of which *A. fumigatus*, *A. flavus*, *A. niger* or *A. terreus* are most commonly found responsible for human disease. *Aspergillus* spp. are widely present in soil, food and moist environments and are known to have a worldwide distribution [74]. Spores (called conidia) are abundantly distributed in the air and can cause various respiratory diseases following inhalation. Exposure from the environment is difficult to preclude, with the exception of hospital rooms equipped with HEPA-filters [75]. Invasive pulmonary aspergillosis is a disease associated with high mortality but only occurs in immunocompromised patients. In contrast, the more chronic forms of pulmonary aspergillosis, e.g., aspergilloma and allergic broncho-pulmonary aspergillosis (ABPA), are found in patients without severe immune deficiency [76, 77]. The lungs or the rhino-sinusal cavities are the primary location of invasive infection in 90-95% of cases. Via the blood circulation *Aspergillus* can metastasize to other organs, dissemination to the central nervous system being one of the complications most feared [78, 79].

Recognition as one of the most important infectious complications following hematopoietic stem cell transplantation is the driving force of the clinical and experimental research related to *Aspergillus* in general and the pathogenesis of invasive aspergillosis in particular. Although sufficient clinical and biochemical markers are now seemingly available to prevent or identify invasive aspergillosis, the current incidence of disease has stabilized at approximately 5-8% of all patients subject to allogeneic stem cell transplantation [14].



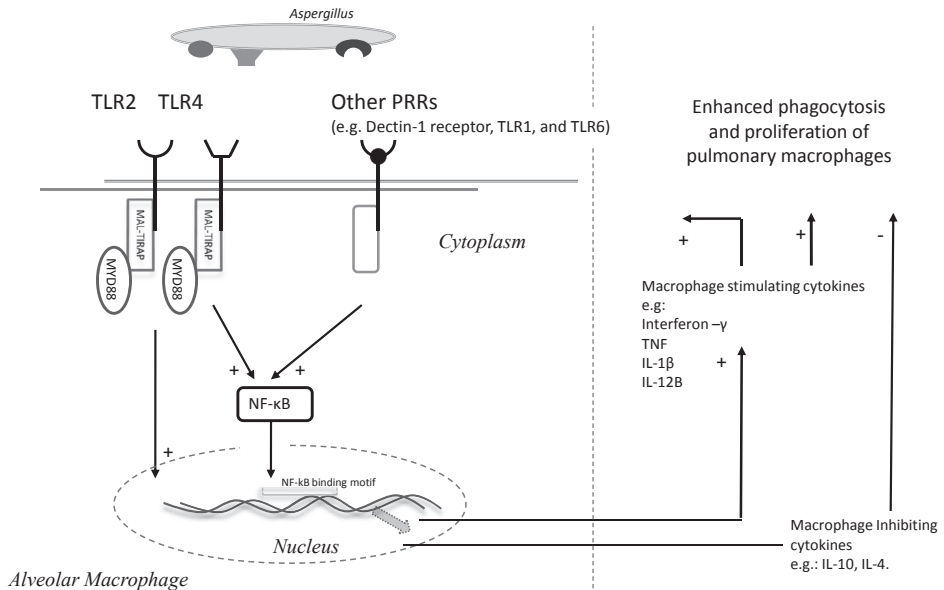
### *Clinical risk factors*

Neutropenia has been designated as the most important risk factor for invasive pulmonary aspergillosis [80]. Clinical characteristics defining risk for development of invasive aspergillosis in patients with haematological diseases were subsequently identified in several studies [81-83]. Overall, older age, presence of graft versus host disease (GVHD), Cytomegalovirus virus infection, use of steroids, recurrence of the underlying disease, and prolonged neutropenia are most strongly associated with acquisition of invasive aspergillosis [84, 85]. With altered transplantation practices, the impact of each of these risk variables for invasive aspergillosis modulate over time [86]. Based on these risk factors, national and international guidelines now assist in the selection of patients with hematological disorders for whom chemoprophylaxis for invasive aspergillosis is indicated [87]. Nevertheless, it has remained incompletely understood why some patients with haematological diseases develop invasive aspergillosis while others remain unaffected. Hence, the risks imposed by exposure, underlying disease, other infectious complications and treatment are not to be considered absolute. In patients with non-haematological underlying disorders (e.g., solid organ transplant recipients), which represent a minority of patients at risk, it is largely unknown which variables predict the development of invasive pulmonary aspergillosis [88, 89].

### *Innate immunity and defense against Aspergillus*

Next to the acknowledged importance of the cellular immunity in the form of neutrophilic granulocytes and - to a lesser extent - specific T-cells, an increasing number of in-vitro and animal studies pointed to the relevance of the innate immune response [90]. Antigen sensing and activation of appropriate host defences by dendritic cells and alveolar macrophages is a pivotal step in the host defence against *Aspergillus* [90]. Transmembrane receptors, including Toll-like receptors and C-type lectin receptors, initiate this process by recognition of the fungus and activates signalling pathways that lead to an inflammatory response (figure 2).

The Toll receptor was originally discovered in *Drosophila sp.* and appeared to play a major role in this organism's defence against fungi [91]. Multiple Toll-like receptors (TLRs) were subsequently identified in humans. TLRs are expressed on the surface of dendritic cells and alveolar macrophages and contain an extracellular domain with leucine-rich repeats and a cytoplasmic Toll/interleukin-1 receptor domain [92]. This latter domain activates common signalling pathways and modulates the expression of genes encoding cytokines and inflammatory molecules. Research in murine models and experimental in-vitro studies pointed to the relevance of TLR2 and TLR4 mediated anti-fungal responses in humans [93, 94]. Furthermore, the overall response of the innate immune system to *Aspergillus* depends on a complex network of activated components encompassing pathogen recognition receptors as well as molecules of the intracellular pathways like MyD88, NFκB and subsequently secreted cytokines [95]. A small number of studies showed that depletion of IL-12, IL-18, TNF



**Figure 2.** Recognition of fungi and fungal pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) 2 and 4 and other pathogen recognition receptors (PRRs) leads to the activation of protective antifungal macrophage responses. The common signaling pathway for mammalian Toll-like receptors (TLRs) and other PRRs involves interaction with the adaptor molecule MYD88 (myeloid differentiation primary response gene 88) located in the cytoplasm. The activation of the MYD88 adaptor eventually results in the activation and nuclear translocation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and subsequent gene transcription of modulating cytokine genes. Depending on the stimulus, enhanced phagocytosis and proliferation of macrophages as well as further activation or deprivation of the cellular immunity will occur.

(tumor necrosis factor) and IFN- $\gamma$  delayed pulmonary clearance of *Aspergillus fumigatus* in mice [96]. In addition, high production of IL-12 and IFN- $\gamma$  acted protective [97]. In vivo - i.e., in the transplant recipient developing invasive aspergillosis - knowledge about the exact role of these TLRs and cytokines in the context of macrophage-related innate anti-fungal defense mechanisms is limited. The components of innate immunity may become trivial in the absence of neutrophilic granulocytes. In this setting, reduced functioning of a TLR or other pattern recognition receptor and impairment in the downstream chain of signalling molecules and cytokines may constitute an important additional risk factor for the acquisition of invasive aspergillosis.

### Diagnosis of invasive aspergillosis

The European Organization for Research and Treatment of Cancer and Invasive Fungal Infections Cooperative Group's revised definitions of invasive fungal disease, categorizes the diagnosis of invasive aspergillosis (and other invasive mycosis) in 3 levels of certainty [98]. The definitions 'proven', 'probable' and 'possible' established a formal context that allowed the identification of more or less homogeneous groups of patients for clinical and epidemiologic research. Expanding knowledge of the molecular biology and immunology has led to the development of diagnostic tests that detect cell wall components of *Aspergillus* in serum or

BAL fluid [99, 100]. The commercial galactomannan and  $\beta$ -D-glucan assays are now widely used for both screening and diagnosis of invasive aspergillosis during neutropenia. Although concerns with regard to sensitivity and specificity do exist, these tests attribute to earlier diagnosis and subsequently improved survival [101]. Noteworthy, new tests that rely on quantification of circulating *Aspergillus* specific T-cells or PCR methods are being developed and assessed in clinical practice for usefulness and reliability with regard to the diagnosis of invasive aspergillosis [102].

### ***Pneumocystis* pneumonia and Invasive Aspergillosis following Transplantation: Indicators of Transmission, Risk and Disease.**

In general, no curative therapy matches the effects of prevention. Of the four major preventive medical strategies: immunization (I), behavioral counseling (II), screening for early stages of disease or screening for risk factors for disease (III) and chemoprevention (IV), the latter two in particular apply to the prevention (or early diagnosis) of invasive fungal infection of the lungs. In both infections with *Pneumocystis* and *Aspergillus* the prognosis depends on the timing of diagnosis. Therefore, reliable indicators of disease, or even better: well described clinical and biochemical markers that flag the need for selective interventional chemoprophylactic strategies or pre-emptive treatment, are required.

For *Pneumocystis* pneumonia, the elucidation of the clinical epidemiology and mode(s) of transmission together with more accurate definition of the clinical risk factors in the non-HIV infected hosts would enable more efficient, selective prescription of chemoprophylaxis and other measures of prevention. Furthermore, there is an urgent need for improving the diagnostic tools by development and implementation of non-invasive tests to establish or to rule out a diagnosis of PCP.

In the advanced research field of invasive pulmonary aspergillosis unraveling of the role of innate immunity precedes the next question (investigated in many other areas of medicine) on how certain genetic mutations, or the individual genetic signature as a whole, influences the likelihood for developing disease in the context of other risk factors. Answers to this question may potentially lead to more sophisticated and effective selection of patients at risk and subsequent prevention by chemoprophylaxis or optimized screening strategies that enable the start pre-emptive treatment.

The research described in this thesis focuses on:

- Analysis of the potential mode(s) of transmission of *P. jirovecii* during an outbreak of PCP
- Identification of risk factors for fungal infection in transplant recipients by case control studies:
  - a) Clinical risk factors in kidney transplant recipients for development of PCP

- b) Genetic risk factors in allogeneic stem cell transplant recipients for development of invasive aspergillosis
- Exploration of potential selective (i.e. individualized) chemoprophylactic strategies for prevention of PCP in transplant recipients
- The prospective assessment of the diagnostic utility of new serum markers for the diagnosis of PCP in the HIV-negative immunocompromised host.
- Evaluation of currently available radiolabeled tracers for future use as specific markers for fungal infection.

## Outline of the Thesis

### Part I

*Pneumocystis* in kidney transplant recipients: transmission, risk factors, new diagnostic and chemo-prophylactic strategies.

**Chapter 2** describes the characteristics of a large outbreak of *Pneumocystis* pneumonia among kidney transplant recipients. By performing a classical outbreak investigation and by application of new molecular genotyping techniques, the potential of the 'interhuman transmission hypothesis' is addressed and discussed.

In **Chapter 3** all currently available data on reported outbreaks of *Pneumocystis* pneumonia is systematically reviewed with the emphasis on mortality data, clinical risk factors and transmission analyses.

In the case-control study described in **Chapter 4**, we performed a detailed risk factor analysis for development of PCP in kidney transplant recipients and used the multivariate output data to estimate the effects of several chemoprophylactic strategies by modeling the expected incidence and number-needed-to-treat to provide efficient PCP chemoprophylaxis over a 2-year period post transplantation.

**Chapter 5** reports the data of a prospective study on the serum markers S-adenosylmethionine and (1→3)-β-D-glucan serum levels and correlation with clinical parameters in HIV-negative immunocompromised patients – the majority kidney transplant recipients – with *Pneumocystis* pneumonia. Potential applicability for treatment monitoring and assessment of *P. jirovecii* pulmonary load is also discussed.

## **Part II**

*Genetic predisposition for development of invasive aspergillosis in stem cell transplant recipients*

**Chapter 6** describes a multicenter study on the impact of the Y238X stop mutation in the human Dectin-1 receptor (which senses and attaches to glucan moieties of the fungal cell wall) on the risk of development of invasive aspergillosis in stem cell transplant recipients.

In **Chapter 7** a retrospective study of the influence of genetic variation in the macrophage activation route with respect to the relative additional risk for development of invasive aspergillosis is presented.

## **Part III**

*Experimental markers for detection of fungal infection: scintigraphic imaging.*

In **Chapter 8** the clinical applicability of radiolabeled antimicrobial peptides and antifungal drugs for the diagnosis of invasive fungal infections is reviewed, together with a concise discussion about how promising agents should be further developed.

The results of the thesis are summarized and discussed in **Chapter 9**.

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