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 hypheal 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 hematopoetic 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

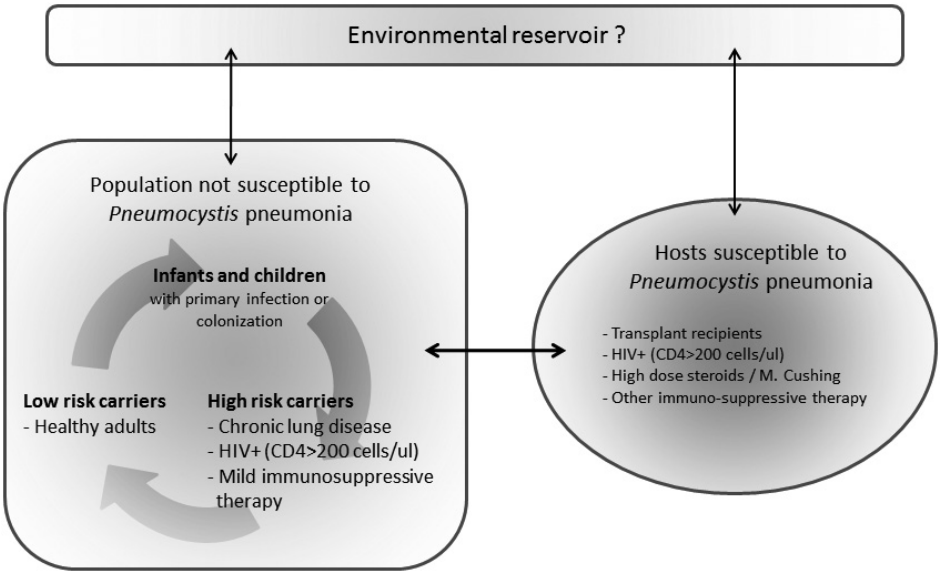
The *Pneumocystis* genus consists of multiple individual species that each require a specific mammalian host [17]. Accompanying this selective infectivity, morphological and biological differences between separate species have been demonstrated. At the electron microscopic level, individual *Pneumocystis* species show distinctive morphology of the filopodia as well as different densities of the membrane-limited cytoplasmatic 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 endogenic 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* naïve, 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 (β-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-kB activation, since a single deficiency of one of the recognition 'molecules' did not exclude an effective activation of this pathway [52-54]. NF-kB 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-γ in particular is noteworthy because of its ability to induce macrophage activation and to exert inflammatory effects by T-cells. Neutralization of IFN-γ 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 immunoflorescent 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 cytoplasmatic 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

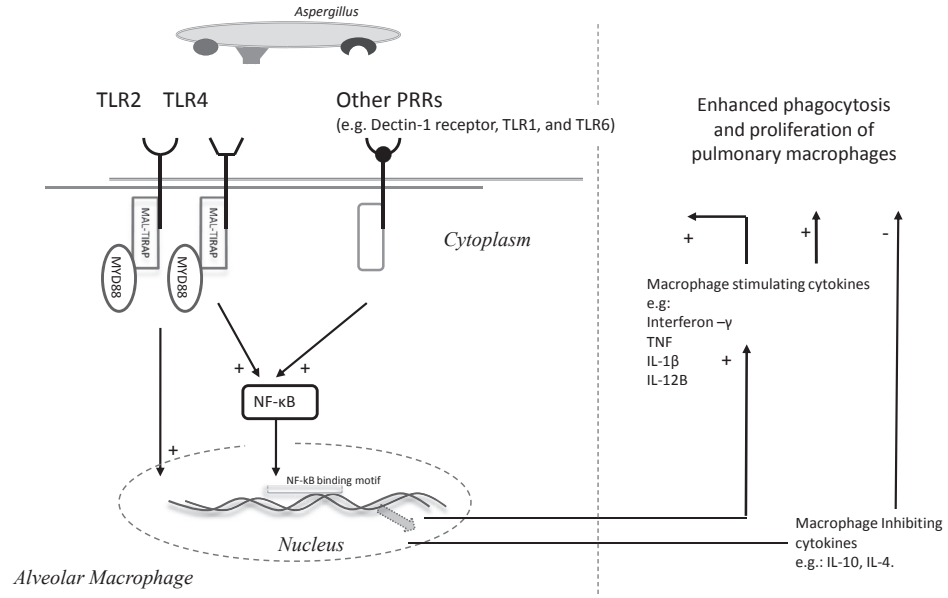


Figure 2. Recognition of fungi and fungal pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) 2 and 4 and other pathogen recognition receptors (PRRs) lead to the activation of protective antifungal macrophage responses. The common signaling pathway for mammalian Toll-like receptors (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-κB (NF-κB) 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-γ delayed pulmonary clearance of *Aspergillus fumigatus* in mice [96]. In addition, high production of IL-12 and IFN-γ 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 β-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 Transplantion: 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**.

References

1. Zimmerman L. Fatal fungus infections complicating other diseases. Am J Clin Pathol 1955; 25(1): 46-65.
2. Gajdusek D. Pneumocystis carinii; etiologic agent of interstitial plasma cell pneumonia of premature and young infants. Pediatrics 1957; 19(4 Part 1):543-65.
3. Winston DJ, Gale RP, Meyer DV, Young LS. Infectious complications of human bone marrow transplantation. Medicine (Baltimore) 1979; 58(1):1-31.
4. The UCLA Bone Marrow Transplantation Group. Bone marrow transplantation with intensive combination chemotherapy/radiation therapy (SCARI) in acute leukemia. Ann Intern Med 1977; 86(2):155-61.
5. Stake G, Flatmark A. Lung complications during immunosuppressive treatment in renal transplant recipients. Scand J Respir Dis 1976; 57(2):51-62.
6. White DA, Santamauro JT. Pulmonary infections in immunosuppressed patients. Curr Opin Pulm Med 1995; 1(3):202-8.
7. Gottlieb MS, Groopman JE, Weinstein WM, Fahey JL, Detels R. The acquired immunodeficiency syndrome. Ann Intern Med 1983; 99(2):208-20.
8. Luce JM. The acquired immunodeficiency syndrome. A report on the Second International Conference on AIDS. Am Rev Respir Dis 1986; 134(5):859-61.
9. Buchacz K, Baker RK, Palella FJ, Jr., Chmiel JS, Lichtenstein KA, et al. AIDS-defining opportunistic illnesses in US patients, 1994-2007: a cohort study. AIDS 2010; %19;24(10):1549-59.
10. Green H, Paul M, Vidal L, Leibovici L. Prophylaxis for Pneumocystis pneumonia (PCP) in non-HIV immunocompromised patients. Cochrane Database Syst Rev 2007;(3):CD005590.
11. Hughes WT, Kuhn S, Chaudhary S, Feldman S, Verzosa M, et al. Successful chemoprophylaxis for Pneumocystis carinii pneumonitis. N Engl J Med 1977; 297(26):1419-26.
12. Branten AJ, Beckers PJ, Tiggeler RG, Hoitsma AJ. Pneumocystis carinii pneumonia in renal transplant recipients. Nephrol Dial Transplant 1995; 10(7):1194-7.
13. Garcia-Vidal C, Upton A, Kirby KA, Marr KA. Epidemiology of invasive mold infections in allogeneic stem cell transplant recipients: biological risk factors for infection according to time after transplantation. Clin Infect Dis 2008; 47(8):1041-50.
14. Kontoyiannis DP, Marr KA, Park BJ, Alexander BD, Anaissie EJ, et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clin Infect Dis 2010; 50(8):1091-100.
15. Neofytos D, Fishman JA, Horn D, Anaissie E, Chang CH, et al. Epidemiology and outcome of invasive fungal infections in solid organ transplant recipients. Transpl Infect Dis 2010; 12(3):220-9.
16. Anaissie EJ. Trial design for mold-active agents: time to break the mold--aspergillosis in neutropenic adults. Clin Infect Dis 2007; 44(10):1298-306.
17. Wakefield AE, Stringer JR, Tamburrini E, Dei-Cas E. Genetics, metabolism and host specificity of Pneumocystis carinii. Med Mycol 1998; 36 Suppl 1:183-93.:183-93.
18. Nielsen MH, Settnes OP, Aliouat EM, Cailliez JC, Dei-Cas E. Different ultrastructural morphology of Pneumocystis carinii derived from mice, rats, and rabbits. APMIS 1998; 106(8):771-9.

19. Dei-Cas E, Mazars E, Ferragut CO, Durand I, Aliouat EM, et al. Ultrastructural, genomic, isoenzymatic and biological features make it possible to distinguish rabbit Pneumocystis from other mammal Pneumocystis strains. J Eukaryot Microbiol 1994; 41(5):84S.

20. Cushion MT, Stringer JR. Has the name really been changed? It has for most researchers. Clin Infect Dis 2005; 41(12):1756-8.

21. Hughes WT. Pneumocystis carinii versus Pneumocystis jirovecii (jiroveci) Frenkel. Clin Infect Dis 2006; 42(8):1211-2.

22. Kaneshiro ES, Ellis JE, Jayasimhulu K, Beach DH. Evidence for the presence of "metabolic sterols" in Pneumocystis: identification and initial characterization of Pneumocystis carinii sterols. J Eukaryot Microbiol 1994; 41(1):78-85.

23. Stringer SL, Stringer JR, Blase MA, Walzer PD, Cushion MT. Pneumocystis carinii: sequence from ribosomal RNA implies a close relationship with fungi. Exp Parasitol 1989; 68(4):450-61.

24. Bartlett MS, Vermund SH, Jacobs R, Durant PJ, Shaw MM, et al. Detection of Pneumocystis carinii DNA in air samples: likely environmental risk to susceptible persons. J Clin Microbiol 1997; 35(10): 2511-3.

25. Hughes WT. Natural mode of acquisition for de novo infection with Pneumocystis carinii. J Infect Dis 1982; 145(6):842-8.

26. Keely SP, Baughman RP, Smulian AG, Dohn MN, Stringer JR. Source of Pneumocystis carinii in recurrent episodes of pneumonia in AIDS patients. AIDS 1996; 10(8):881-8.

27. Vargas SL, Hughes WT, Santolaya ME, Ulloa AV, Ponce CA, et al. Search for primary infection by Pneumocystis carinii in a cohort of normal, healthy infants. Clin Infect Dis 2001; 32(6):855-61.

28. Meuwissen JH, Tauber I, Leeuwenberg AD, Beckers PJ, Sieben M. Parasitologic and serologic observations of infection with Pneumocystis in humans. J Infect Dis 1977; 136(1):43-9.

29. Durand-Joly I, Soula F, Chabe M, Dalle JH, Lafitte JJ, et al. Long-term colonization with Pneumocystis jirovecii in hospital staffs: a challenge to prevent nosocomial pneumocystosis. J Eukaryot Microbiol 2003; 50 Suppl:614-5.

30. Ponce CA, Gallo M, Bustamante R, Vargas SL. Pneumocystis colonization is highly prevalent in the autopsied lungs of the general population. Clin Infect Dis 2010; 50(3):347-53.

31. Medrano FJ, Montes-Cano M, Conde M, de la HC, Respaldiza N, et al. Pneumocystis jirovecii in general population. Emerg Infect Dis 2005; 11(2):245-50.

32. Vargas SL, Ponce CA, Sanchez CA, Ulloa AV, Bustamante R, Juarez G. Pregnancy and asymptomatic carriage of Pneumocystis jiroveci. Emerg Infect Dis 2003; 9(5):605-6.

33. Peterson JC, Cushion MT. Pneumocystis: not just pneumonia. Curr Opin Microbiol 2005; 8(4): 393-8.

34. Thomas CF, Jr., Limper AH. Current insights into the biology and pathogenesis of Pneumocystis pneumonia. Nat Rev Microbiol 2007; 5(4):298-308.

35. Opportunistic Infections Project Team of the Collaboration of Observational HIV Epidemiological Research. Is it safe to discontinue primary Pneumocystis jiroveci pneumonia prophylaxis in patients with virologically suppressed HIV infection and a CD4 cell count <200 cells/microL? Clin Infect Dis 2010; 51(5):611-9.

36. Mansharamani NG, Balachandran D, Vernovsky I, Garland R, Koziel H. Peripheral blood CD4 + T-lymphocyte counts during Pneumocystis carinii pneumonia in immunocompromised patients without HIV infection. Chest 2000; 118(3):712-20.

37. Arend SM, Westendorp RG, Kroon FP, van't Wout JW, Vandenbroucke JP, et al. Rejection treatment and cytomegalovirus infection as risk factors for Pneumocystis carinii pneumonia in renal transplant recipients. Clin Infect Dis 1996; 22(6):920-5.

38. Arichi N, Kishikawa H, Mitsui Y, Kato T, Nishimura K, et al. Cluster outbreak of Pneumocystis pneumonia among kidney transplant patients within a single center. Transplant Proc 2009; 41(1): 170-2.

39. Bjorklund A, Aschan J, Labopin M, Remberger M, Ringden O, et al. Risk factors for fatal infectious complications developing late after allogeneic stem cell transplantation. Bone Marrow Transplant 2007; 40(11):1055-62.

40. Nevez G, Raccurt C, Vincent P, Jounieaux V, Dei-Cas E. Pulmonary colonization with Pneumocystis carinii in human immunodeficiency virus-negative patients: assessing risk with blood CD4+ T cell counts. Clin Infect Dis 1999; 29(5):1331-2.

41. Nevez G, Raccurt C, Jounieaux V, Dei-Cas E, Mazars E. Pneumocystosis versus pulmonary Pneumocystis carinii colonization in HIV-negative and HIV-positive patients. AIDS 1999; 13(4):535-6.

42. Roifman CM, Hummel D, Martinez-Valdez H, Thorner P, Doherty PJ, et al. Depletion of CD8+ cells in human thymic medulla results in selective immune deficiency. J Exp Med 1989; 170(6):2177-82.

43. Gigliotti F, Wright TW. Immunopathogenesis of Pneumocystis carinii pneumonia. Expert Rev Mol Med 2005; 7(26):1-16.

44. Pop SM, Kolls JK, Steele C. Pneumocystis: immune recognition and evasion. Int J Biochem Cell Biol 2006; 38(1):17-22.

45. Gigliotti F, Wright TW. Immunopathogenesis of Pneumocystis carinii pneumonia. Expert Rev Mol Med 2005; 7(26):1-16.

46. Pop SM, Kolls JK, Steele C. Pneumocystis: immune recognition and evasion. Int J Biochem Cell Biol 2006; 38(1):17-22.

47. Steele C, Marrero L, Swain S, Harmsen AG, Zheng M, et al. Alveolar macrophage-mediated killing of Pneumocystis carinii f. sp. muris involves molecular recognition by the Dectin-1 beta-glucan receptor. J Exp Med 2003; 198(11):1677-88.

48. Linke MJ, Ashbaugh AD, Demland JA, Walzer PD. Pneumocystis murina colonization in immunocompetent surfactant protein A deficient mice following environmental exposure. Respir Res 2009; 10:10.

49. Zhang C, Wang SH, Lasbury ME, Tschang D, Liao CP, et al. Toll-like receptor 2 mediates alveolar macrophage response to Pneumocystis murina. Infect Immun 2006; 74(3):1857-64.

50. Ding K, Shibui A, Wang Y, Takamoto M, Matsuguchi T, Sugane K. Impaired recognition by Toll-like receptor 4 is responsible for exacerbated murine Pneumocystis pneumonia. Microbes Infect 2005; 7(2):195-203.

51. Zhang C, Wang SH, Liao CP, Lasbury ME, Durant PJ, et al. Toll-like receptor 2 knockout reduces lung inflammation during Pneumocystis pneumonia but has no effect on phagocytosis of Pneumocystis organisms by alveolar macrophages. J Eukaryot Microbiol 2006; 53 Suppl 1:S132-3.: S132-S133.

52. Chen W, Havell EA, Moldawer LL, McIntyre KW, Chizzonite RA, Harmsen AG. Interleukin 1: an important mediator of host resistance against Pneumocystis carinii. J Exp Med 1992; 176(3):713-8.

53. Lebron F, Vassallo R, Puri V, Limper AH. Pneumocystis carinii cell wall beta-glucans initiate macrophage inflammatory responses through NF-kappaB activation. J Biol Chem 2003; 278(27): 25001-8.

54. Wang SH, Zhang C, Lasbury ME, Liao CP, Durant PJ, et al. Decreased inflammatory response in Toll-like receptor 2 knockout mice is associated with exacerbated Pneumocystis pneumonia. Microbes Infect 2008; 10(4):334-41.

55. Rudmann DG, Preston AM, Moore MW, Beck JM. Susceptibility to Pneumocystis carinii in mice is dependent on simultaneous deletion of IFN-gamma and type 1 and 2 TNF receptor genes. J Immunol 1998; 161(1):360-6.

56. Lasbury ME, Durant PJ, Ray CA, Tschang D, Schwendener R, Lee CH. Suppression of alveolar macrophage apoptosis prolongs survival of rats and mice with pneumocystis pneumonia. J Immunol 2006; 176(11):6443-53.

57. Limper AH, Hoyte JS, Standing JE. The role of alveolar macrophages in Pneumocystis carinii degradation and clearance from the lung. J Clin Invest 1997; 99(9):2110-7.

58. Peterson JC, Cushion MT. Pneumocystis: not just pneumonia. Curr Opin Microbiol 2005; 8(4): 393-8.

59. Azoulay E, Bergeron A, Chevret S, Bele N, Schlemmer B, Menotti J. Polymerase chain reaction for diagnosing pneumocystis pneumonia in non-HIV immunocompromised patients with pulmonary infiltrates. Chest 2009; 135(3):655-61.

60. Morris A, Wei K, Afshar K, Huang L. Epidemiology and clinical significance of pneumocystis colonization. J Infect Dis 2008; 197(1):10-7.

61. Alvarez-Martinez MJ, Miro JM, Valls ME, Moreno A, Rivas PV, et al. Sensitivity and specificity of nested and real-time PCR for the detection of Pneumocystis jiroveci in clinical specimens. Diagn Microbiol Infect Dis 2006; 56(2):153-60.

62. Shimizu Y. Serum markers in interstitial pneumonia with and without Pneumocystis jirovecii colonization: a prospective study. 2009.

63. Jarowenko M, Pifer L, Kerman R, Kahan BD. Serologic methods for the early diagnosis of Pneumocystis carinii infection in renal allograft recipients. Transplantation 1986; 41(4):436-42.

64. Lundgren B, Lundgren JD, Nielsen T, Mathiesen L, Nielsen JO, Kovacs JA. Antibody responses to a major Pneumocystis carinii antigen in human immunodeficiency virus-infected patients with and without P. carinii pneumonia. J Infect Dis 1992; 165(6):1151-5.

65. Skelly MJ, Holzman RS, Merali S. S-adenosylmethionine levels in the diagnosis of Pneumocystis carinii pneumonia in patients with HIV infection. Clin Infect Dis 2008; 46(3):467-71.

66. Tasaka S, Hasegawa N, Kobayashi S, Yamada W, Nishimura T, et al. Serum indicators for the diagnosis of pneumocystis pneumonia. Chest 2007; 131(4):1173-80.

67. Yasuoka A, Tachikawa N, Shimada K, Kimura S, Oka S. (1-->3) beta-D-glucan as a quantitative serological marker for Pneumocystis carinii pneumonia. Clin Diagn Lab Immunol 1996; 3(2): 197-9.

68. von Eiff M, Roos N, Wilms B, Walger P, Baumgart P, et al. [Pneumocystis carinii pneumonia in HIV-positive and HIV-negative patients]. Schweiz Rundsch Med Prax 1990; 79(18):569-73.

69. Ewig S, Bauer T, Schneider C, Pickenhain A, Pizzulli L, et al. Clinical characteristics and outcome of Pneumocystis carinii pneumonia in HIV-infected and otherwise immunosuppressed patients. Eur Respir J 1995; 8(9):1548-53.

70. Ziefer A, Abramowitz JA. Pneumocystis carinii pneumonia in HIV-positive and HIV-negative patients. An epidemiological, clinical and histopathological study of 18 patients. S Afr Med J 1989; 76(7):308-13.

71. Limper AH, Offord KP, Smith TF, Martin WJ. Pneumocystis carinii pneumonia. Differences in lung parasite number and inflammation in patients with and without AIDS. Am Rev Respir Dis 1989; 140(5):1204-9.

72. Su YS, Lu JJ, Perng CL, Chang FY. Pneumocystis jirovecii pneumonia in patients with and without human immunodeficiency virus infection. J Microbiol Immunol Infect 2008; 41(6):478-82.

73. Del Bono V, Mularoni A, Furfaro E, Delfino E, Rosasco L, et al. Clinical evaluation of a (1,3)-beta-D-glucan assay for presumptive diagnosis of Pneumocystis jiroveci pneumonia in immunocompromised patients. Clin Vaccine Immunol 2009; 16(10):1524-6.

74. Klich MA. Health effects of Aspergillus in food and air. Toxicol Ind Health 2009; 25(9-10):657-67.

75. Hahn T, Cummings KM, Michalek AM, Lipman BJ, Segal BH, McCarthy PL, Jr. Efficacy of high-efficiency particulate air filtration in preventing aspergillosis in immunocompromised patients with hematologic malignancies. Infect Control Hosp Epidemiol 2002; 23(9):525-31.

76. Riscili BP, Wood KL. Noninvasive pulmonary Aspergillus infections. Clin Chest Med 2009; 30(2): 315-35, vii.

77. Agarwal R. Allergic bronchopulmonary aspergillosis. Chest 2009; 135(3):805-26.

78. Scully EP, Baden LR, Katz JT. Fungal brain infections. Curr Opin Neurol 2008; 21(3):347-52.

79. Ruhnke M, Kofla G, Otto K, Schwartz S. CNS aspergillosis: recognition, diagnosis and management. CNS Drugs 2007; 21(8):659-76.

80. Latge JP. Aspergillus fumigatus and aspergillosis. Clin Microbiol Rev 1999; 12(2):310-50.

81. Pagano L, Caira M, Candoni A, Offidani M, Martino B, et al. Invasive aspergillosis in patients with acute myeloid leukemia: a SEIFEM-2008 registry study. Haematologica 2010; 95(4):644-50.

82. Avery RK. Aspergillosis in hematopoietic stem cell transplant recipients: risk factors, prophylaxis, and treatment. Curr Infect Dis Rep 2009; 11(3):223-8.

83. Mikulska M, Raiola AM, Bruno B, Furfaro E, Van Lint MT, et al. Risk factors for invasive aspergillosis and related mortality in recipients of allogeneic SCT from alternative donors: an analysis of 306 patients. Bone Marrow Transplant 2009; 44(6):361-70.

84. Marr KA, Carter RA, Boeckh M, Martin P, Corey L. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. Blood 2002; 100(13):4358-66.

85. Fukuda T, Boeckh M, Carter RA, Sandmaier BM, Maris MB, et al. Risks and outcomes of invasive fungal infections in recipients of allogeneic hematopoietic stem cell transplants after nonmyeloablative conditioning. Blood 2003; 102(3):827-33.

86. Garcia-Vidal C, Upton A, Kirby KA, Marr KA. Epidemiology of invasive mold infections in allogeneic stem cell transplant recipients: biological risk factors for infection according to time after transplantation. Clin Infect Dis 2008; 47(8):1041-50.

87. Stevens DA, Kan VL, Judson MA, Morrison VA, Dummer S, et al. Practice guidelines for diseases caused by Aspergillus. Infectious Diseases Society of America. Clin Infect Dis 2000; 30(4):696-709.

88. Cornillet A, Camus C, Nimubona S, Gandemer V, Tattevin P, et al. Comparison of epidemiological, clinical, and biological features of invasive aspergillosis in neutropenic and nonneutropenic patients: a 6-year survey. Clin Infect Dis 2006; 43(5):577-84.

89. Patterson JE, Peters J, Calhoon JH, Levine S, Anzueto A, et al. Investigation and control of aspergillosis and other filamentous fungal infections in solid organ transplant recipients. Transpl Infect Dis 2000; 2(1):22-8.

90. Shomah S, Levitz SM. The immune response to fungal infections. Br J Haematol. 2005; 129(5): 569-82.

91. Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 1996; 86(6): 973-83.

92. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004; 4(7):499-511.

93. Braedel S, Radsak M, Einsele H, Latge JP, Michan A, et al. Aspergillus fumigatus antigens activate innate immune cells via toll-like receptors 2 and 4. Br J Haematol 2004; 125(3):392-9.

94. Van der Graaf C, Kullberg BJ, Joosten L, Verver-Jansen T, Jacobs L, et al. Functional consequences of the Asp299Gly Toll-like receptor-4 polymorphism. Cytokine 2005; 30(5):264-8.

95. Lasker MV, Nair SK. Intracellular TLR signaling: a structural perspective on human disease. J Immunol 2006; 177(1):11-6.

96. Brieland JK, Jackson C, Menzel F, Loebenberg D, Cacciapuoti A, et al. Cytokine networking in lungs of immunocompetent mice in response to inhaled Aspergillus fumigatus. Infect Immun 2001; 69(3):1554-60.

97. Cenci E, Mencacci A, Fe dC, Del Sero G, Mosci P, et al. Cytokine- and T helper-dependent lung mucosal immunity in mice with invasive pulmonary aspergillosis. J Infect Dis 1998; 178(6):1750-60.

98. De PB, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 2008; 46(12):1813-21.

99. Leeflang MM, Debets-Ossenkopp YJ, Visser CE, Scholten RJ, Hooft L, et al. Galactomannan detection for invasive aspergillosis in immunocompromized patients. Cochrane Database Syst Rev 2008;(4):CD007394.

100. Maertens J, Theunissen K, Lodewyck T, Lagrou K, Van EJ. Advances in the serological diagnosis of invasive Aspergillus infections in patients with haematological disorders. Mycoses 2007; 50 Suppl 1:2-17.:2-17.

101. Upton A, Gugel A, Leisenring W, Limaye A, Alexander B, et al. Reproducibility of low galactomannan enzyme immunoassay index values tested in multiple laboratories. J Clin Microbiol 2005; 43(9):4796-800.

102. Potenza L, Barozzi P, Vallerini D, Bosco R, Quadrelli C, et al. Diagnosis of invasive aspergillosis by tracking Aspergillus-specific T cells in hematologic patients with pulmonary infiltrates. Leukemia 2007; 21(3):578-81.