Risk factors and new markers of pulmonary fungal infection

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Pneumocystis pneumonia and Invasive Aspergillosis following Transplantion:
Indicators of Transmission, Risk and Disease

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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

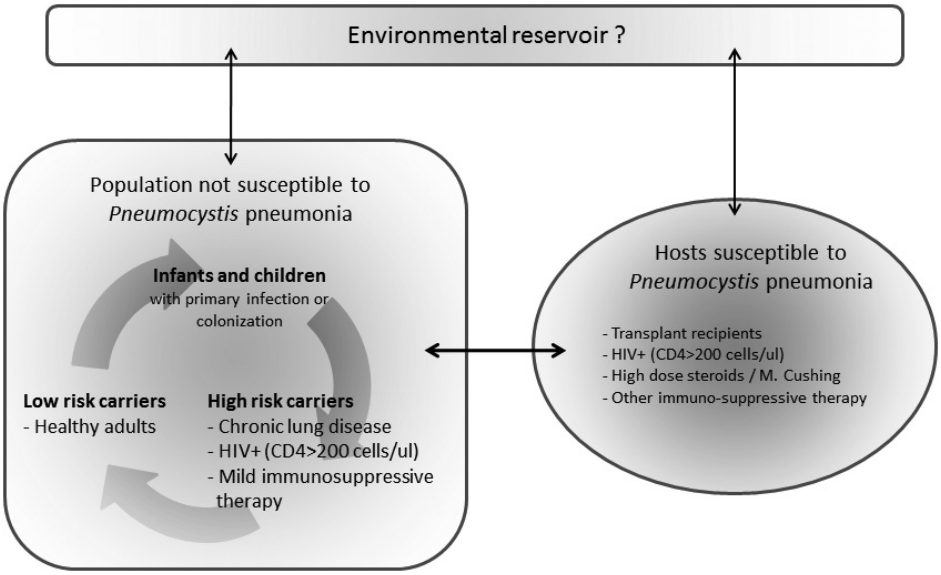
The *Pneumocystis* genus consists of multiple individual species that each require a specific mammalian host [17]. Accompanying this selective infectivity, morphological and biological differences between separate species have been demonstrated. At the electron microscopic level, individual *Pneumocystis* 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

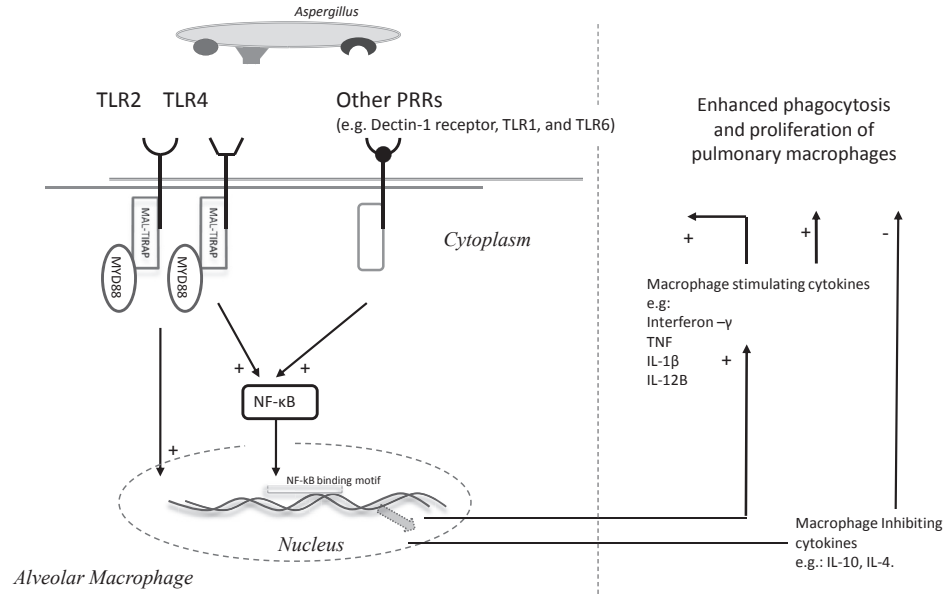


Figure 2. Recognition of fungi and fungal pathogen-associated molecular patterns (PAMPs) by Toll-like receptors (TLRs) 2 and 4 and other pathogen recognition receptors (PRRs) lead to the activation of protective antifungal macrophage responses. The common signaling pathway for mammalian Toll-like receptors (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**.

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Part I

PNEUMOCYSTIS PNEUMONIA IN KIDNEY TRANSPLANT RECIPIENTS: TRANSMISSION, RISK FACTORS, NEW DIAGNOSTIC AND CHEMOPROPHYLACTIC STRATEGIES

Chapter 2

AN OUTBREAK OF *PNEUMOCYSTIS JIROVECII* PNEUMONIA WITH ONE PREDOMINANT GENOTYPE IN RENAL TRANSPLANT RECIPIENTS: INTERHUMAN TRANSMISSION OR A COMMON ENVIRONMENTAL SOURCE?

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Abstract

Background: An outbreak of *Pneumocystis* pneumonia (PCP) in renal transplant recipients attending the outpatient department occurred in the Leiden University Medical Centre between the first of March 2005 and the first of February 2006; clinical, epidemiological and molecular characteristics were analysed to trace its origin.

Methods: Renal transplant recipients with a clinical suspected diagnosis of PCP were included. The diagnosis had to be confirmed by direct microscopy or real time PCR of the dihydropteroate synthase (DHPS) gene in broncho-alveolar fluid. To detect contacts between patients a transmission map was constructed. A case-control analysis was performed to asses whether infection was associated with certain wardrooms. Genotyping of *Pneumocystis* was performed by sequence analysis of the internal transcribed spacer number 1 (ITS1) and ITS2 gene regions.

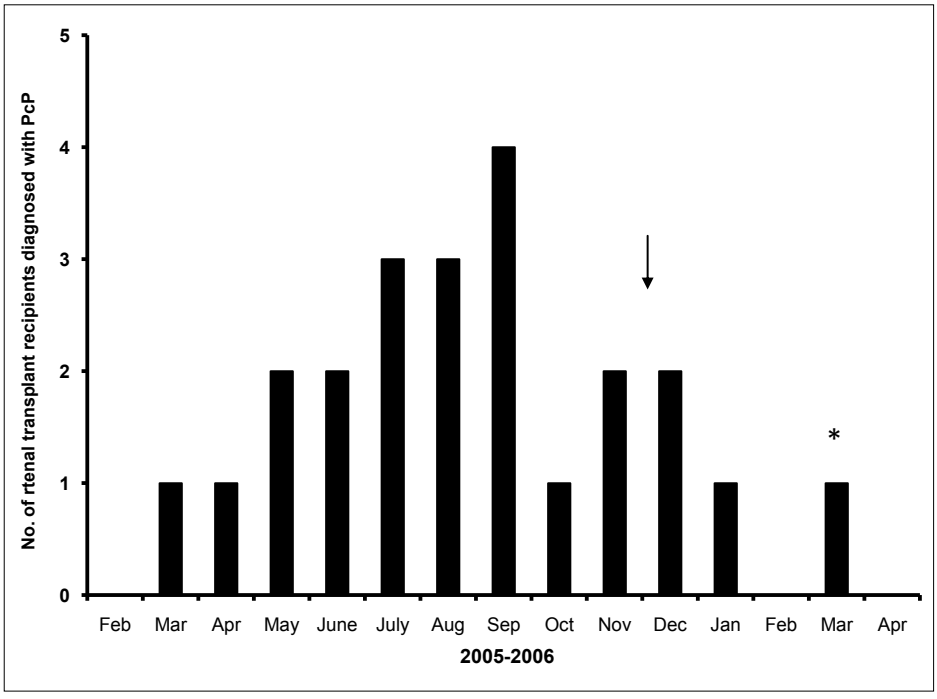
Results: 22 confirmed PCP cases were identified; about 0 to 1 would have been expected over the same time period. No risk factor was predominantly present and standard immune-suppressive regimens had not changed. Liver transplant recipients using the same outpatient facilities had not acquired PCP. The transmission map was compatible with interhuman transmission on multiple occasions. The case-control study did not point to wardrooms as a common source. Genotyping by sequencing of the ITS1 and ITS2 gene regions showed type 'Ne' in 12 out of 16 successfully typed samples. Genotype 'Ne' was found in only 2 out of 12 reference samples.

Conclusions: The clinical data and genotyping are compatible with either interhuman transmission or an environmental source; more complex models may account for PCP clusters.

Introduction

Pneumocystis pneumonia (PCP), caused by *Pneumocystis jirovecii*, remains a substantial cause of morbidity and mortality in immuno-compromised individuals [1]. The development of animal models and genotyping methods has contributed to an increased understanding of the complex behaviour of this opportunistic pathogen [2, 3]. However, the exact mode of transmission and acquisition of this saprophytic infection are still unclear. Different sources of infection have been proposed, e.g. the environment or asymptomatic carriers [4, 5]. Recently, the possible role of interhuman transmission between immuno-compromised patients was described [6-8]. In this article we report an outbreak of PCP in a population of renal transplant recipients attending the outpatient post-transplantation department of the the Leiden University Medical Center (LUMC) between the first of March 2005 and the first of February 2006. PCP was diagnosed in 22 renal transplant recipients (figure 1). In our transplant program ±100 patients receive a kidney or kidney-pancreas transplant each year; specialized care is provided for about one thousand renal transplant recipients. In this population the expected incidence of PCP is 0 to 1 case per year as estimated from registration data from the departments of microbiology and infectious diseases from 1995 onwards. Because of the sudden rise in incidence and possible contact between patients when visiting the nephrol-

Figure 1. Number of renal transplant recipients with confirmed PCP in 2005-2006 per month; * marks the last case, reported after February 1st 2006. The arrow indicates the start of antibiotic prophylaxis for PCP.



ogy outpatient department, either interhuman transmission or a local environmental source was suspected. The clinical, epidemiological and molecular characteristics of this outbreak were analysed by conducting five separate investigations (descriptive epidemiology, statistical analysis of outpatient contacts, a case-control study, air sampling and genotyping of *Pneumocystis* strains) to elucidate its origins. We discuss the results along with two currently proposed models of transmission of *P. jirovecii*.

Methods

Patient data

All renal transplant recipients presenting with dyspnoea and interstitial pneumonia in which the diagnosis of PCP was considered, were included. The time window of the study ranged between the first of March 2005 and the first of February 2006. After the beginning of the outbreak, nephrologists and microbiologists in hospitals participating in our transplant program were requested to report patients with a renal transplant and interstitial pneumonia. The diagnosis of PCP was regarded confirmed if *P. jirovecii* was detected by direct microscopy (Silver- and Giemsa staining) or real time PCR of the dihydropteroate synthase (DHPS) gene in bronchoalveolar lavage (BAL) fluid [9]. Data about underlying disease, immune suppressive medication, use of PCP prophylaxis, dates of hospital visits and demographical data were obtained from the files. The clinical presentation of PCP was briefly described. A transmission map was constructed to detect contacts between patients during admittances to the nephrology unit and visits to the nephrology outpatient department. Two nephrologists (A.G. and S.P.B.) verified that there had been no changes in immune-suppressive regimens. PCP prophylaxis was not prescribed routinely.

Statistical analysis of outpatient department contacts

A separate analysis was performed on the transmission map data to assess whether a patient who had received the diagnosis of PCP on a particular day had more often visited the outpatient department in the four months preceding the diagnosis in comparison with patients who would become diseased later. Also it was assessed whether a patient that was diagnosed with PCP on a particular day, had more frequently encountered other future patients (i.e., potentially contagious patients) in the outpatient department in comparison to patients who would only become diseased later. These analyses were performed with a Cox model wherein the time varying exposure were the number of visits and the number of potentially infected patients with whom a patient had contact before the onset of disease.

Case-control study for inpatient rooms (Nephrology Unit)

Because the majority of the patients had been hospitalised before the PCP outbreak, we investigated the possibility of transmission via a common source located in – or nearby – rooms of the nephrology unit by means of a case-control analysis. Cases were defined as renal transplant recipients with confirmed PCP in 2005 who had stayed in the nephrology unit earlier in 2005, i.e. before the diagnosis of PCP. The control group consisted out of renal transplant recipients admitted to the unit in the same time window but who were not diagnosed with PCP later. Data were obtained from the hospital's administrative department. Odds ratios and 95%-confidence intervals were calculated for all rooms.

Air sampling

Air sampling was performed to detect *Pneumocystis* in rooms of the nephrology unit and in the waiting room of the outpatient department. Because this expertise was not available in our institution, the collection of air samples and the procedure of extracting DNA from the filters were carried out by a company specialised in measuring microbiological air quality (Intersave Groeneveld B.V., Dordrecht, The Netherlands). The following locations were sampled: a wardroom of the nephrology unit, the nurse post of the nephrology unit and the outpatient department waiting room (twice). Also a room in the hospital that was never used for patient care, and a room that was used by a patient with PCP (a supposed negative and positive control room) were sampled. The outpatient department was sampled overnight when no patients were present. Air sampling was performed by use of Gilair air sampler pumps (Sensidyne Inc., Florida, USA.), creating an airflow over a glass fiber filter with a velocity of 2 liters per minute for ±8 hours on each location. After filtration of approximately 1000 litres of air, the filters were removed and DNA was extracted (Chemagic DNA extraction kit, Chemagen, Baesweiler, Germany). Specimens were transported to the laboratory of the microbiology department of the LUMC for analysis by real time PCR (DHPS gene). Further investigations included the analysis of multi-layered filters of the ventilation system of the outpatient department. Two filters passed by inflowing air and one outflow filter were sampled by cutting a 10 cm^2 piece of each filter, which was washed with 500 ml of MilliQ and centrifuged. Both supernatant and residue were subjected to real time PCR (DHPS gene).

Genotyping of Pneumocystis strains

Genotyping of *P. jirovecii* was performed by sequence analysis of the internal transcribed spacer number 1 (ITS1) and ITS2 of the nuclear rRNA operon. Reference data reflecting the distribution of *P. jirovecii* genotypes in this region was obtained by genotyping 11 samples obtained from patients with PCP admitted to the LUMC between January 2003 and January

2005 and 3 samples containing *P. jirovecii* from other Dutch hospitals (all not related to this outbreak).

The forward primer (ITS1F) was described previously by Lu et al.[10, 11]. The reverse primer (ITS2R1) from Lu et al. was shortened and used with the sequence 5'-GCGGGTGATCCTGCCT-3' to lower the melting temperature. The formed PCR product consists of the ITS1, 5.8S gene and the ITS2 gene region and has a total length of approximately 540 bp. DNA was extracted from BAL samples using the total nucleic acid protocol with the MagNA pure LC nucleic acid isolation system (Roche Diagnostics, Almere, The Netherlands). Each sample was eluted in 100 µl of buffer and stored at –80°C until processing. 5 µl of DNA-extract was added to 45 µl reaction mix containing 25 µl of 2x Hotstar mastermix (Qiagen, Venlo, The Netherlands) and 25 pmol of each primer. Cycling conditions: 15 min at 95°C, 50 cycles of 30 s at 92°C, 30 s at 62°C, and 30 s at 72°C respectively, followed by a 5 min. hold at 72°C. The PCR product was analysed with agarose gel electrophoresis. In case of aspecific amplification, the correct product was cut out and purified using the Qia-quick gel-extraction kit (Qiagen). Sequencing was performed on an ABI3100 automatic sequencer (Applied Biosystems) using a sequencing ready reaction kit (ABI). Sequence types were designated according to the method of Lee et al. [12].

Results

Patient characteristics and outcome

Twenty-six patients presenting with symptoms and radiological signs compatible with PCP were identified. The diagnosis of PCP was confirmed in 22 cases; 16 with positive microscopy and PCR, 1 with microscopy and 6 with PCR only. Twelve (55%) were male; age ranged from 36 to 72 years (median 57). No geographic clustering according to postal code was noted. All patients (including the 6 patients reported from other hospitals) except one had visited the nephrology outpatient department of the LUMC. The cause of original renal disease was heterogeneous; 3 out of 22 cases had received a kidney-pancreas transplant and 11 cases had received their graft within 1 year prior to the diagnosis of PCP. Immune-suppressive regimens contained mofetyl mycofenolate and prednisone (7.5 to 20 mg once daily) in all but one patient. Ten patients also used cyclosporine. No changes in routine immune-suppressive regimens had been implemented in the past 5 years. Although aware of the recommendation of the European guidelines [13], it was the nephrology department's policy - prior to this outbreak - not to prescribe trimethoprim-sulfamethoxazole prophylaxis. Because the very low incidence of PCP so far, the benefits were not considered to outweigh the side effects.

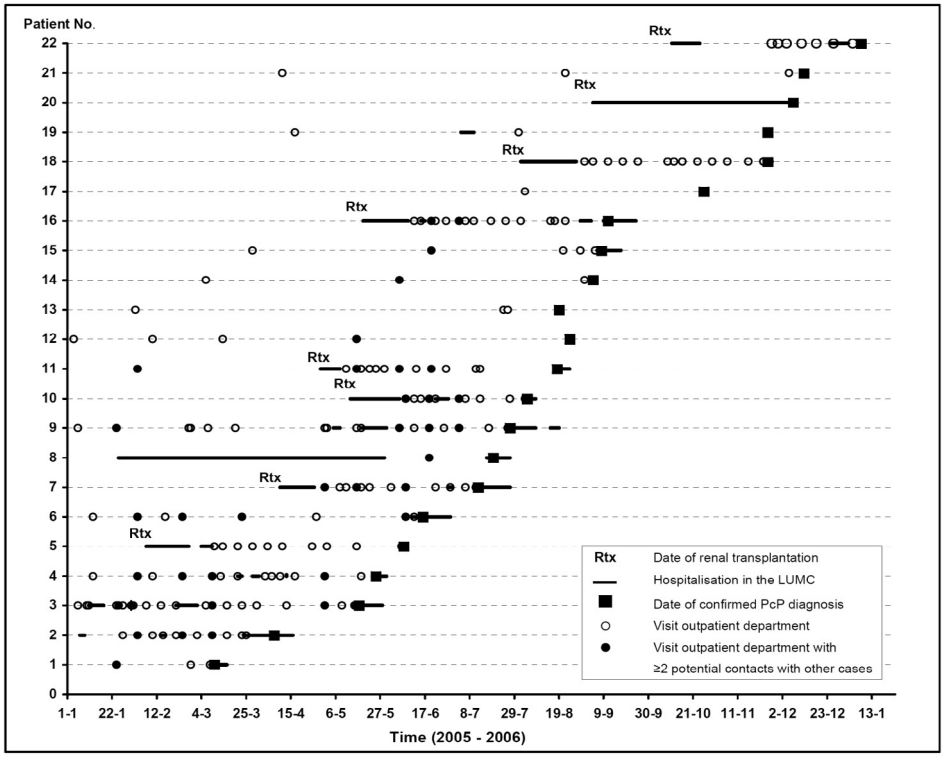
Cytomegalovirus (CMV) replication was present in 10 of 19 patients with known CMV status; only one received anti-viral medication at the time of diagnosis. Five patients had received treatment for graft rejection within 12 months before the diagnosis of PCP.

One patient became critically ill and died due to pulmonary and cardiac failure; none of the other patients was transferred to an intensive care unit.

Transmission map

The transmission map (figure 2) showed that interhuman transmission of *Pneumocystis* might have been possible on multiple occasions during outpatient department visits. The map does not allow to define a moment that all patients were in contact. However, if time windows are taken in to account, multiple possibilities of transmission exist. When each case is regarded as a possible index case, a combination of patient No.3 and No.9 suffices to trace potential contacts with all but one case with type 'Ne' (the predominant genotype). Both patients had received multiple treatments for rejection and had a higher load of *Pneumocystis* in BAL fluid (microscopy 3+, Ct values 34.4 and 27.5 respectively) in comparison to other patients.

Figure 2. Transmission map.



Legend: Genotyping of *Pneumocystis* showed ITS type 'Ne' in cases 2,3,6,7,9-13,16-18. Only ITS2 could be determined for case 14: 'e'. Determination of ITS genotypes failed in case 1,4,5 and 20-22. Genotype 'Bi' was found in case 8. PCP was diagnosed in this case after a long stay on the hematology ward due to treatment for malignant lymphoma.

Statistical analysis of outpatient department contacts

An analysis of the frequency of visits to the outpatient department, and the encounter of other future patients over the four months preceding the PCP diagnosis was performed on the transmission chart data (i.e. on cases only). The frequency of visit was more strongly associated with disease development than encounter of other patients who developed PCP at a later time (i.e., potentially contagious patients).

Case-control analysis

There were several time windows in which 2 or more patients of this cluster had been admitted to the nephrology unit at the same time before they had developed PCP. In the case-control study on inpatient rooms, a total of 24 and 257 hospitalisations of 10 case patients and 139 control patients were analysed (data not shown). The odds ratios for individuals rooms and for combinations of rooms varied from 0.75 to 1.89 with 95% confidence intervals including 1.00.

Air sampling

This part of the study was conducted in January and February 2006. The mean total amount of filtered air on each location was 1000 liters (range 861-1216 liters). In none of the 6 samples derived from the pump filters *Pneumocystis* was detected by real time PCR. The supposedly positive control room was found to be negative. The negative results were not due to inhibition of the samples since the internal controls (phocine herpes virus) were positive. A specimen derived from one of the inlet-filters of a ventilation shaft tested positive for PCP. Subsequent ITS genotyping failed probably due to sequence homology with ITS eukaryotic plant material. The outlet filters tested negative.

Genotyping of Pneumocystis strains

Identification of *Pneumocystis* strains by genotyping of the ITS1 and ITS2 gene regions was accomplished in 16 of the 22 available BAL samples from 22 different patients. Sequence analysis showed type 'Ne' in 12 out of 16 successfully analysed samples, type 'Bi' was present in 1 sample. In 3 samples only the ITS2 genotypes could be determined: type 'e' once and 'g' twice. Genotyping failed in 6 samples due to a weak signal or the presence of >2 strains. Interestingly, of the 12 successfully genotyped reference samples (i.e. unrelated to the present outbreak) only 2 (17%) showed type 'Ne'.

Discussion

Unique aspects of this outbreak of PCP are the relatively large number of cases, the high probability of contact between cases and the observation of one predominant *P. jirovecii* genotype. From the first of December 2005 - the 10th month of the outbreak - trimethoprim-sulfamethoxazole or an alternative form of prophylaxis was prescribed for patients within their first year post transplantation as well as for patients treated for graft rejection. Despite increased alertness among physicians, only one new case was reported since February 2006. We found no indication that the incidence of PCP had increased due to changes in immune-suppressive medication. The risk of PCP is associated with the treatment for rejection and CMV reactivation [14, 15], but none of these risk factors was predominantly present or changed over time and thus unlikely to have played a major role. We discuss the results of this outbreak investigation along with two hypothetical models of transmission of *P. jirovecii*.

The environmental source hypothesis

This thesis is based on the assumption that inhaled forms of *P. jirovecii* that cause PCP do directly originate from a niche in the environment. No environmental source has been discovered to harbour *P. jirovecii* in previous outbreaks. Air sampling studies indicated that the route of transmission is by air [16, 17]. However, the source from which *P. jirovecii* becomes airborne remains to be specified: either environmental or human. Attempts to isolate *Pneumocystis* by air sampling were unsuccessful in this study; the sensitivity and specificity of the methods used are unknown. The positive PCR analysis of one of the *inlet* filters is compatible with more than one hypothesis about transmission, but suggests an environmental source.

The epidemiological data neither exclude nor suggest the presence of an environmental source. The communal presence of patients in the outpatient department can equally indicate that they acquired PCP through interhuman transmission as that they were infected by a local environmental source. No geographic clustering by postal code was noted, making one or multiple regional environmental source(s) outside the hospital less likely.

The statistical approach - the analysis of outpatient visit frequency and frequency of encounter of other future patients in the outpatient department – showed the strongest association with the number of times a patient visited the outpatient department. This points to an environmental source. However, the almost constant presence of several future patients and the unknown incubation time makes it uncertain whether these calculations can reliably discriminate between interhuman transmission and an environmental source.

Genotyping showed that 12(75%) of genotyped strains were *P. jirovecii* type 'Ne'. Analysis of the reference strains indicates that type 'Ne' is less frequent in this region. However, in Denmark type 'Ne' was the second most prevalent strain in a study of randomly selected *P. jirovecii* positive BAL samples, but was not found in a cluster reported from that country [12,

18]. Data from Thailand and England confirm a regional dependent distribution of *P. jirovecii* ITS types [19, 20]. Since the frequency of different ITS types of *P. jirovecii* in the Leiden region is unknown, definite interpretation of the genotyping results is not possible. Genotype 'Ne' has not been reported as a more virulent type in human disease [21].

The interhuman transmission hypothesis

In this model PCP is considered a transmittable disease; i.e. the source is another infected (or colonized) individual [22]. Accumulating evidence from animal models, the host specificity of *P. jirovecii* and the phenomenon of carriage of *P. jirovecii* in the respiratory tract of healthy and immuno-compromised individuals all support the idea that humans may constitute the reservoir themselves [3, 23-26]. Transmission would then occur by spreading and subsequent inhalation of air or aerosols containing infectious forms of *P. jirovecii*. Four previous studies attempted to elucidate the possible role of interhuman transmission in small clinical clusters of PCP by genotyping (summarized in table 1). The methods used and the clinical evidence of contact between patients differs between these studies. In the two more recent investigations, genotyping and clinical data suggest the possibility of interhuman transmission [8, 28].

The transmission map from our study shows that contact between cases was possible on multiple occasions in this outbreak. Although details of interactions between patients

Table 1. Studies of recent clinical clusters of *Pneumocystis* pneumonia in which genotyping was performed.

Authors (ref.)	Year of publication	No. of patients (clustered)	Clinical background	Genotyping method	Authors' conclusions
Helweg-Larsen J. et al. [17]	1998	14 (8+4+3)	8 Clustered cases of patients with an haematological malignancy and PCP plus 2 small clusters of HIV infected individuals	DNA sequence analysis ITS1, ITS2	Interhuman transmission may occur but may not constitute the major route of transmission.
Ollson et al. [27]	2001	17 (3+7+7)	3 Clusters, 2 in renal transplant recipients, 1 in patients with a haematological malignancy.	DNA sequence analysis mtLSU-rRNA	Interhuman transmission unlikely
Rabodorina M. et al. [8]	2004	10	A cluster of PCP in renal transplant recipients who encountered HIV+ patients during hospital admittance	multitarget PCR-SSCP ITS1, 26S, mt26, beta-tubulin	Possible nosocomial interhuman transmission
Hocker B. et al. [26]	2005	6 (3+3)	Sudden rise in incidence of PCP in a paediatric transplant unit; 3 cases on a clinical and molecular basis related to one index case	multitarget PCR-SSCP ITS1, 26S, mt26, beta-tubulin	Possible nosocomial interhuman transmission

SSCP denotes single strand confirmation polymorphism, ITS1: internal transcribed spacer 1, 26S intron of the Nuclear 26S rRNA gene, mt26: variable region of the Mitochondrial 26S rRNA gene, beta tubulin: region surrounding intron 6 of the beta-tubulin gene, mtLSU-rRNA: mitochondrial large subunit ribosomal RNA locus, HIV: Human Immuno-deficiency Virus, PCP: *Pneumocystis* pneumonia.

could not be reconstructed, it accurately describes the presence of one or more cases within a limited waiting area (16m^2) within a limited time period. In view of the fact that the actual mechanism of transmission of *P. jirovecii* is unknown, the thesis that one or two 'hyper-spreaders' (case No.3 and No.9) caused this outbreak remains speculative.

Interestingly, the outbreak was restricted to renal transplant recipients. No case of PCP was found in the population of 200 liver transplant recipients in our hospital, despite the fact that these patients wait in the same waiting room as the renal transplant recipients. The number of hospital visits in both populations is proportional but visiting hours overlap just one morning per week. Because the liver transplant recipients use comparable immune-suppressive drugs and do not receive PCP prophylaxis, a proportional incidence of up to 4 cases of PCP would be expected in case of an environmental source in the outpatient department [29]. The interpretation of the genotyping results - potentially supportive for both hypotheses - is discussed in the previous paragraph.

Interpretation and conclusions

In this outbreak the evidence for clinical clustering as well as the presence of one predominant genotype are compatible with either an environmental source or interhuman transmission. From published data one can not assess whether clustering of PCP usually starts with index patients, comes from a temporary increased reservoir of carriers in the general population or from an environmental source. Hence, the need for further elucidation of the general mode of transmission of *P. jirovecii* seems evident. This requires sophisticated methods to investigate possible environmental sources and clinical research aimed at understanding the role of carriers of *P. jirovecii*. More complex transmission models may account for clusters of PCP. Progress in understanding the source of PCP may permit to undertake effective action to prevent and control future outbreaks.

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Chapter 3

OUTBREAKS AND CLUSTERING OF *PNEUMOCYSTIS* PNEUMONIA IN KIDNEY TRANSPLANT RECIPIENTS: A SYSTEMATIC REVIEW

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Submitted

Abstract

From 1980 onwards, an increasing number of outbreaks of *Pneumocystis* pneumonia (PCP) among kidney transplant recipients have been reported. The cause of these outbreaks is unclear and different explanations have been provided. We performed a systematic review to provide a comprehensive overview of the epidemiologic characteristics as well as the involved clinical risk factors. A total of 15 peer reviewed English language articles published from 1980 onward were included. Outbreak settings were all marked by absence of chemoprophylaxis, frequent inter-patient contacts and lack of isolation measures taken during hospitalization of PCP cases. PCP associated mortality rates significantly decreased from a weighted mean of 38% before 1990 to 19% and 13% in the following two decades. Clinical risk factors for PCP in outbreaks settings were largely similar to non-outbreak settings. Genotyping by multilocus sequence typing (MLST) or comparison of the internal transcribed spacer (ITS) regions 1 and 2 showed that the outbreaks are most frequently caused by a predominant or a single *Pneumocystis* strain. Pooled epidemiological data and genotyping results strongly attest to the emerging insight that acquisition of *Pneumocystis* occurs through interhuman transmission. No seasonal trend was noted. The results emphasize the need for chemoprophylaxis in kidney transplant recipients despite a low baseline incidence of PCP in this population and support the current CDC recommendation with regard to isolation of patients with PCP during hospitalization.

Introduction

Pneumocystis pneumonia (PCP), caused by *Pneumocystis jirovecii*, has been recognized as an important potential cause of morbidity and mortality in patients susceptible due to immunosuppressive medication needed after kidney or other (solid organ) transplantation [1]. Interestingly, and apart from the expected observation of solitary cases, an increasing number of sudden outbreaks or clusters* of PCP among kidney transplant recipients was reported from several continents. The pathogenesis of these outbreaks has not been clarified and different explanations e.g. changes in the standard immunosuppressive regimen, an environmental source or patient-to-patient transmission have been proposed [2-4]. Recent outbreaks occurred in the absence of chemoprophylaxis, while in general the prescription of Trimethoprim-Sulfamethoxazole (TMP-SMX) to prevent PCP for at least a duration of 3-6 months after kidney transplantation now is a widely accepted practice and incorporated in several kidney transplantation guidelines [5, 6].

During the first observations of clusters of PCP in kidney transplant units in the 1980s, where transplant recipients were hospitalized together with AIDS patients, the possibility of patient-to-patient transmission and a relation with the developing HIV epidemic in the northern hemisphere in general was proposed [7]. Outbreaks among kidney transplant recipients and other immunocompromised hosts were thereafter repeatedly observed but analysis was restricted to assessment of the epidemiological data and investigation of potential clinical risk factors [8-11]. The application of molecular genotyping methods from 1990 onwards pointed to the likelihood of a either a common environmental source or interhuman transmission of PCP during an outbreak [12-16]. Untill now an environmental source has not been identified and evidence from several studies demonstrated colonization of the human respiratory tract in asymptomatic healthy and immunocompromised individuals in up to 50% of the studied cases [17-20].

Combining these and other recent findings with the historical data of PCP outbreaks involving kidney transplant recipients, may lead to improved understanding of the factors contributing to *Pneumocystis* transmission and development of outbreaks of PCP among kidney transplant recipients (26). Main objective of this systematic review is to provide a comprehensive overview of the involved clinical risk factors as well as epidemiological characteristics of PCP outbreaks among kidney transplant recipients. Based on the results, we update current opinions with regard to the involved risk factors and mechanism(s) of transmission. This should lead to improved implementation – and maybe new - strategies for prevention of PCP in solid organ transplant recipients.

** When the term 'outbreak' is used, it is considered to represent both the terms 'cluster' and 'outbreak' throughout the manuscript.*

Methods

A systematic literature review was performed to identify all English language papers describing PCP outbreaks in renal transplant recipients from 1980 onwards. An outbreak was defined as a reported unexpected sudden rise in incidence of PCP comprising at least 5 kidney transplant recipients. A comprehensive literature search using the PubMed and Medline databases was performed. The following exact search commands were used in both databases: (I) *'transplantation AND Pneumocystis AND outbreak'*, (II) *'transplantation AND Pneumocystis AND cluster'*, (III) *'kidney transplantation AND Pneumocystis'*. Results were limited to English language articles, adult human subjects and time period from 1980 onward. The search procedure was repeated several times untill the 1st of August 2010. A total of 145 peer reviewed articles were identified. All available abstracts were reviewed by the first author. Of the 32 articles of potential interest 14 fulfilled the inclusion definition. In case of doubt the last author was consulted. The PubMed option ´related articles´, reference lists of included articles and consultation of expert sources were used to find additional articles missed by the initial search. One additional article was included by this secondary search (figure 1).

The following data was obtained from all 15 included articles: the total number of patients, year of publication, year of index case diagnosis, geographical region, PCP incidence prior to the outbreak, overall mortality, outcome of nested risk factor analysis, outcome of genotyping analysis performed on *Pneumocystis* organisms (if available) and environmental investigations. In table 1 (page 75) the major characteristics of the included outbreaks are described. A meta-analysis was not attempted due to the heterogeneity of transmission analyses. In the sections below the results and observations are discussed in the context of the current knowledge about *P. jirovecii*.

Figure 1. Summary of systematic literature search results.

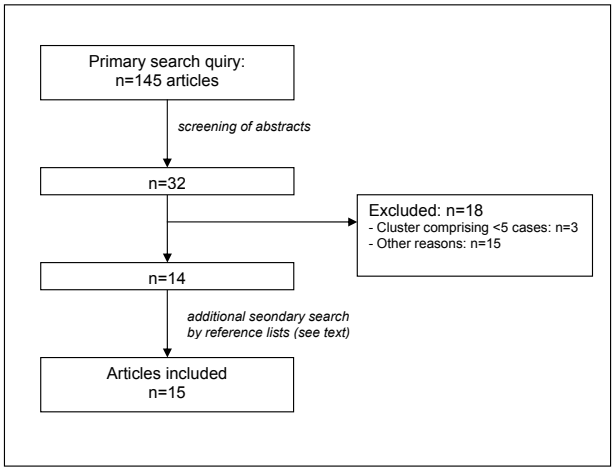


Table 1. Included *Pneumocystis* outbreaks in kidney transplant recipients 1980-2010.

No., 1st Author (ref)	Publication (year)	Geographic location	Index case (year)	N	<1year post transplantation (%)	Risk analysis (case-control)	Genotyping of *Pneumocystis*	Reported mortality (%)
1- Hardy [22]	1984	Pittsburg, USA	1982	14	86	Yes	No	21.4
2- Santiago-Delpin [3]	1988	San Juan, Peurto Rico	1985	11	NA	No	No	45.5
3- Talseth [4]	1988	Oslo, Norway	1985	14	100	No	No	50.0
4- Bensousan [8]	1990	Brest, France	1990	6	NA	No	No	NA
5- Chave [7]	1991	Lausanne Switzerland	1988	5	40	No	No	NA
6- Branten [9]	1995	Nijmegen, Netherlands	1991	28	NA	Yes	No	21.4
7- Hennequin [10]	1995	Paris, France	1995	7	86	No	No	42.9
8- Lufft [11]	1996	Hannover, Germany	1993	7	NA	No	No	0
9- Olsson [50]	2001	Solna, Sweden	1987/1988	5+7†	NA	No	Yes	NA
10- Rabodorina [13]	2004	Lyon, France	1994	10	50	No	Yes	10.0
11- de Boer [2]	2007	Leiden, Netherlands	2005	22	50	No	Yes	4.5
12- Schmoldt [15]	2008	Munchen, Germany	2006	16	94	No	Yes	25.0
13- Yazaki [14]	2009	Nagoya, Japan	2004	27	41	No	Yes	3.7
14- Arichi [16]	2009	Hyogo, Japan	2007	9	44	Yes	No	33.3
15- Gianelli [12]	2009	Zurich, Switzerland	2006	20	55	No	Yes	15.0

Legend: NA denotes that information was not available; † 2 separate clusters were described.

Results and Discussion

Descriptive epidemiology

A total of 16 outbreaks, described in 15 articles, were identified and comprised a median number of 12 cases, with a range up to 28 cases. In the first reports of clusters of PCP in kidney transplant units in the 1980s, the rise of PCP incidence was linked to the rapidly expanding solid organ transplanted population and the introduction of Cyclosporine as a maintenance drug to prevent graft rejection [3, 21]. Overlapping hospitalization of PCP cases was already noted but absence of molecular methods only allowed speculation about the possibility of interhuman transmission [3, 4, 22]. In one study a case-control investigation strongly indicated transmission of PCP from HIV-infected patients to kidney transplant recipients [7]. At the time, this observation was seen as support for the hypothesis that the overall rising incidence of PCP due to the HIV epidemic influenced the risk for PCP in other immunocompromised populations [23]. More extended mapping of potential interhuman transmission occasions was performed in later outbreak studies. These detailed descriptions all demonstrated the high probability of frequent contact between kidney transplant recipients who developed

PCP. However, this observation may be inherent also to the post transplant state in which patients have to submit to frequent hospital visits. Interestingly, none of the studies report a coincidental increase in PCP incidence in other immunocompromised populations e.g. liver or lung transplant recipients at the same institution. The epidemiological data of the larger outbreaks (n>15, all occurring after 1990) show bell shaped incidence curves, suggesting a common origin resulting from either interhuman transmission or an environmental source.

A period of preceding years with very low prevalence (<2%) of PCP in the kidney transplant population was reported in 75% of the outbreaks. As discussed by many of the authors, this low incidence of PCP had prompted local transplant committees to weigh Trimethoprim-Sulfamethoxazole side effects against overall morbidity and mortality of PCP. In the respective institutions this resulted in a policy endorsing the absence of PCP chemoprophylaxis post kidney transplantation. Furthermore, avoidance of placement of a patient with PCP next to other immunocompromised patients - as currently recommended by the Centers for Disease Control and Prevention (CDC) - was not routinely practiced at the time in any of the outbreak settings [24].

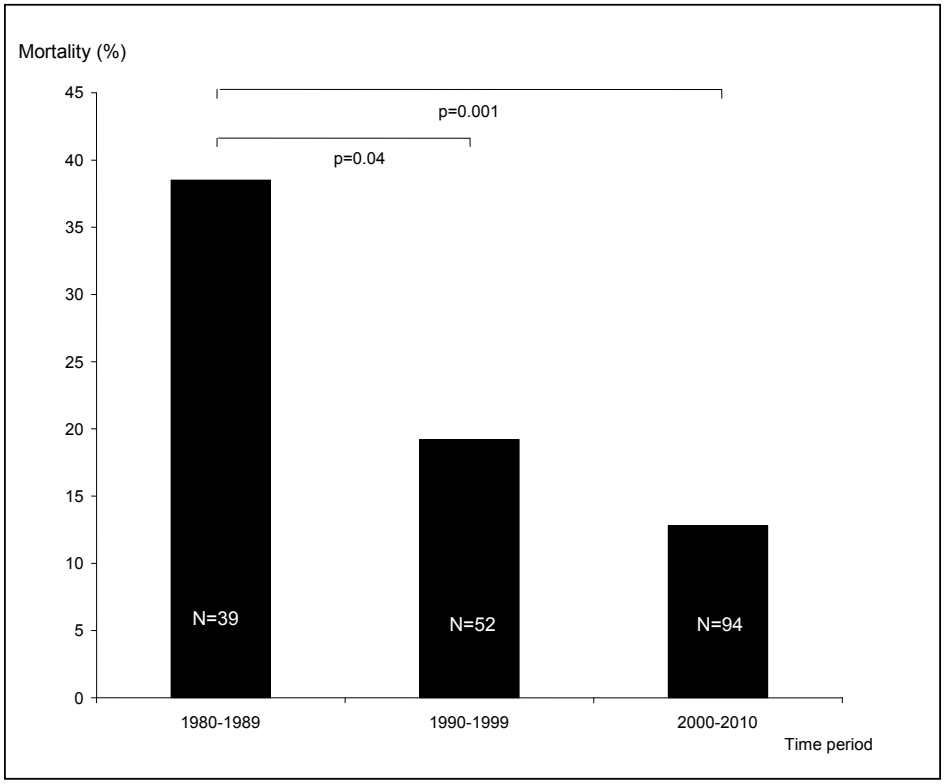
Mortality

Over time, mortality rates in kidney transplant recipients during outbreaks declined from a weighted average of 38% in the 1980s (n=3 studies) to 19% in the 1990s (n=4 studies) and 13% in the first decade of this century (n=5 studies). For 3 studies no mortality data were available. The trend in declining mortality was significant between the first and the last two decades (figure 2). The mortality in HIV-negative immunocompromised patients with PCP has been reported to be relatively high as compared to HIV-positive patients with PCP. In autopsy studies of patients with PCP, the number of Pneumocysts was found to be relatively lower in immunocompromised patients without HIV [25]. In contrast, inflammation was found to be more extensive ref. These observations indicate that not *P. jirovecii* burden but the intensity of the evoked inflammatory reaction determines the severity of disease and the risk of fatal outcome. A number of trials published in the early 1990s demonstrated the beneficial effects of steroids in the treatment of PCP [26-28]. The implementation of conjunct steroid treatment probably accounts for the observed decrease in PCP associated mortality after 1990. In addition, early diagnosis and the availability of high quality intensive care facilities are other important factors that probably attribute to a declining mortality [29].

Clinical risk factors

Only 3 out of the 15 outbreaks explored the potential risk factors by a retrospective case-control investigation. Up to date, only two other retrospective case-control studies that specifically focused on identification of risk factors for PCP in kidney transplant recipients

Figure 2. Histogram showing average Pneumocystis pneumonia associated mortality per decade; N represents the total number of cases from the pooled studies in the respective time period. P-values calculated by Pearson-chi-square exact test.



Legend: N represents the total number of cases from pooled studies per time period. P-values were calculated by Pearson-chi-square test.

not receiving any prophylaxis, were published [30, 31]. Since the included patients in these studies were isolated cases, they were not primarily included for the purpose of this review. Table 2 shows the major findings of the risk factor analyses in two non-outbreak and three outbreak investigations. Patients were most at risk in the first year post transplantation. The weighted average percentage of cases diagnosed within the first year post transplantation was 63% (range: 40% to 100% of cases).

Rejection treatment

A higher frequency of treatment for rejection prior to development of PCP was reported from both non-outbreak studies. In the complete series of outbreak studies any previous treatment for rejection, i.e. high dose steroids or ATG (anti-thymocyte globulin) was present in 22% to 100% of cases (median 50%). Data from 4 outbreak studies specifically reported the use of ATG or OKT3 globulins as rejection treatment in 20 to 39% of cases as compared to 41% and 73% in the two non-outbreak studies. Insufficient data was available to assess

Table 2. Potential individual risk factors for *Pneumocystis* pneumonia in outbreak and non-outbreak settings.

	Non-outbreak studies		Outbreak studies		
Study: 1st author, year	Arend[30] 1995	Radisic[31] 2003	Hardy[22] 1984	Branten[9] 1995	Arichi[16] 2009
No. included cases/controls	15/95	17/34	14/26	28/27	10/431
Mean age: (years)					
cases	48.8	39.7	40.7	50*	46.9*
controls	43.3	35.4	NAβ	38	34.0
Treatment for rejection (any): n (%)					
cases	13 (87)*	17 (100)*	10 (71)	13 (46)	NA
controls	57 (60)	26 (76)	NA	11 (41)	NA
ATG/OKT3 as rejection treatment: n (%)					
cases	11 (73)*	7 (41)*	NA	11 (39)	2 (20)
controls	30 (32)	3 (9)	NA	6 (22)	29 (7)
CMV infection: n (%)					
cases	8 (53)*	9 (53)*	14 (100)*	5 (18)	NA
controls	15 (19†)	8 (24)	13 (65∫)	4 (15)	NA

Legend: NA denotes that information was not available; †: of 80 controls for whom data about CMV was available; ∫: of 20 cases for whom data about CMV was available. β: cases and controls were matched for age in this study. *: significant difference between cases and controls ($p<0.05$).

the role of basiliximab or other immunotherapy's administered at time of transplantation to prevent rejection.

CMV infection

Concurrent CMV infection was associated with development of PCP in both non-outbreak studies as well as in 2 of the 3 case-control investigations within outbreak studies. A high incidence of CMV infection - either reactivation or primary infection - coinciding with PCP was reported in 7 out of the total of 15 outbreak studies. The weighted average percentage of cases with concurrent CMV replication was 63% (range 18% to 100%). Comparisons of these percentages are flawed by changes in the diagnostic methods as well as definition of CMV infection in kidney and other solid organ transplant populations. In addition, new screening and prevention strategies for CMV disease have been implemented over time [32]. It remained unclear whether the association of CMV infection with PCP is due to the underlying gap in T-cell function, putting patients at risk for both infections, or due to the suppressive effect of CMV replication on T-cell function itself. Several clinical studies and in-vitro experiments indicate that CMV has a negative effect on the cellular immune response [33].

Immune suppressive regimen

The occurrence of PCP in renal transplant recipients was directly linked to the immunosuppressive regimen in 4 out of the 15 studies [3, 11, 16, 22]. In two studies the PCP outbreak coincided with the introduction of Cyclosporine A as part of the immunosuppressive therapy [3, 22]. The influence of Cyclosporine A on the risk for PCP was additionally suggested in the publication of case series from several large transplantation centers in the 1980s [21, 34]. However, contradicting observations were reported from case series as well as in one of the outbreak studies [11, 35]. In the case control investigations (of both outbreak and non-outbreak studies) Cyclosporine A based immune suppression was found not to be associated with increased risk for PCP. Experimental studies in rats had previously demonstrated a protective effect of the purine antagonist Mofetyl Mycofenolate against PCP [36]. However, the clinical relevance of this finding was never confirmed through comparative research in humans (16;37;38). In the multivariate analysis performed in the outbreak study performed by Arichi et al., the use of Mofetyl Mycofenolate was even associated with increased risk for PCP [16] .

Molecular epidemiology

In 6 of the 15 included outbreak studies a molecular analysis of the *P. jirovecii* organisms was undertaken to investigate the possibility of interhuman transmission. Table 3 shows the genotyping results of the six PCP outbreak studies among kidney transplant recipients. In 5 of the outbreaks one predominant or a single strain was identified.

Over 14 unique gene loci have been evaluated for the purpose of genotyping applications [37]. The original approach by sequencing of the mitochondrial large subunit (mt LSU) RNA in conjunction with single stranded confirmation polymorphism (SSCP), was used in only 1 of the included studies. After this time newer methods were preferred. Multilocus sequence

Table 3. Genotyping of *Pneumocystis* organisms in 7 outbreaks of *Pneumocystis* pneumonia in kidney transplant recipients.

Study No.	Method	No. of cases included in genotyping analysis / total No. of cases	Genotyping result	Reference group included in the analysis
-9-	Mt LSU RNA +SSCP	3 / 5	Different strains	No
		7 / 7	2 pairs of cases with identical strains	No
-10-	MLST	9 / 10	6 out of 9 strains identical	Yes
-11-	ITS1+ITS2	16 / 22	12 out of 16 strains identical	Yes
-12-	MLST	16 / 16	all strains identical	Yes
-13-	ITS1+ITS2	8 / 27	all strains identical	Yes
-15-	MLST	7 / 7	all strains identical	Yes

Legend: Mt-LSU denotes mitochondrial large subunit ribonucleic acid; MLST: multilocus sequence typing; ITS: internal transcribed spacer region; SSCP: single strand confirmation polymorphism.

typing (MLST) described by Hauser et al. [38], probably has become the most frequently used method and was applied in 3 of the outbreak studies. ITS genotyping, described by Lee et al. [39], was used in 2 of the 6 outbreak studies that applied genotyping methods as well as in several cross sectional epidemiological studies investigating the geographic distribution of *P. jirovecii* genotypes [40, 41]. Interpretation of *P. jirovecii* genotyping results in any outbreak of *Pneumocystis* needs to be performed with care. Several lines of evidence now strongly suggest that infection with *P. jirovecii* occurs through airborne transmission from either patients with overt PCP or individuals – either healthy or immunocompromised – colonized with *P. jirovecii*. This model implicates that genotyping results obtained from an outbreak must not only be linked within the outbreak itself, but should be held also against the background of the circulating genotypes in the population. Background information on circulating strains is necessary to determine whether the outbreak is more likely due to patient-to-patient transmission or increased random transmission from colonized individuals. Secondly, colonization and infection with more than one *P. jirovecii* genotype has been described. The validity of the genotyping methods to detect these double or triple configuration of *Pneumocystis* strains involved is yet unknown and may further complicate interpretation of the genotyping results.

An interpretation of the genotyping results of the outbreak against the background of circulating strains was performed in 5 out of the 6 studies in which genotyping methods were applied. Although reference groups were relatively small (n<50), the genotypes of strains from the control groups were found to be different from those detected in the respective outbreaks. Difficulties in interpretation of the genotyping results due to presence of multiple strains were not reported as a major problem. Variation in virulence between individual *Pneumocystis* strains may have contributed to the genesis of PCP outbreaks. Thus far, investigations failed to indicate such a possible relationship [42].

Environmental investigations

In 2 out of the 15 outbreak studies a local environmental investigation was performed in the hospital. Yazaki et al. [14] performed two sets of environmental surveys using 30-40 swabs in areas visited by affected patients. *P. jirovecii* DNA was found in outpatients consulting rooms. However, the finding could be interpreted as a consequence of the observed outbreak as well as a link to the (environmental) cause. In a study by our group, air samplers were used to collect air specimens during the aftermath of the observed outbreak [2]. From the air filters no *Pneumocystis jirovecii* DNA could be extracted. Though limited by the lack of validated sampling methods, the search for a specific environmental source, during outbreaks and in separate investigations, practically remained without meaningful results [43, 44]. Recently, Choukri et al. published the first report of detection of dispersion of *P. jirovecii* in the air surrounding PCP patients [45]. In this study it was demonstrated that a significant relation existed between the amount of detected *P. jirovecii* DNA and distance from the patient diagnosed with PCP. This

finding further supports the hypothesis that airborne transmission occurs during contact between patients with *Pneumocystis* pneumonia and individuals at risk [45].

Climatic influence

A total of 10 out of the 15 studies contained data about the timing of the cases throughout the year. The timing of peak incidence occurred during winter in 4, during spring in 1 and in summer or early fall in 5 out of 10 studies. The time of diagnosis of the index case was not associated with any month or season. The majority of studies were reported from geographic locations with moderate climate in coastal areas. In epidemiological studies investigating the possible association between overall PCP incidence and climatologic factors from the UK and Spain, a positive correlation was found between PCP incidence and colder months [46-48]. In contrast to these findings, a recent study form Germany, found that higher incidences of PCP were associated with the summer period by using four different climatic factors and the season as variables in a multivariate statistical model [49]. A possible explanation for these different observations may be that other more important factors linked to climate, e.g. human behavior, outweigh the influence of single climatic factors with regard to *Pneumocystis* transmission. As suggested by the study reported by Santiago-Delpin [3], the occurrence of *Pneumocystis* is not restricted to temperate climates only.

Summary and Conclusions

Over time, the descriptive epidemiological and genotyping data of the 15 PCP outbreak studies increasingly pointed to a common source, either environmental or human. This is in contrast to the previous concept of reactivation of *Pneumocystis* in the immunocompromised host, but concurs with the current hypothesis of acquisition of *Pneumocystis* via individuals that are carriers of *P. jirovecii* or that suffer from PCP. Though limited by available methods, the search for a specific environmental source during outbreaks and in separate investigations remained without result. PCP associated mortality rates significantly decreased from a weighted average of 38% before 1990 to less than 20% in the past two decades. Clinical risk factors for PCP during outbreaks were largely similar to non-outbreak settings. Alterations in immune suppressive regimens were at first highly suspected, but never proven to be a major factor in the cause of an outbreak. Treatment for rejection, CMV infection and (potentially) older age were the most important individual risk factors. During the past decades changes occurred with regard to most of these factors. The age limit for solid organ transplantation gradually shifted upwards and more advanced T-cell specific compounds for maintenance immune suppression and treatment as well as prevention of rejection became available. This could have shifted the ratio of kidney transplant recipients at risk versus not at risk for

development of PCP within the kidney transplant population. As a consequence, this would at least facilitate the initiation and propagation of an outbreak. Furthermore, two other important factors permitted the development of the PCP outbreaks. First, the lack of application of droplet isolation measures during hospitalization of kidney transplant recipients with PCP probably increased the exposure of the rest of the population at risk. More importantly, the risk for development of PCP was not negated by the use of chemoprophylaxis for PCP in any of the reported outbreaks settings.

The studies of PCP outbreaks in kidney transplant recipients provide important data that have contributed to the understanding of the mode of transmission and epidemiology of *Pneumocystis*. The discovery of the linkage of each species of *Pneumocystis* to a specific mammalian host and the phenomenon of common asymptomatic carriage in the airways of both healthy and immunocompromised hosts further supports the hypothesis that the human population forms the primary – if not the only - source.

With regard to the data presented in this review, it must be concluded that although PCP incidence in kidney transplant recipients may be low for a prolonged period of time, a chemoprophylactic strategy needs to be implied to prevent both incidental cases and PCP outbreaks. Furthermore, isolation measures must be installed during hospitalization of patients with PCP to prevent transmission to other individuals at risk.

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Chapter 4

RISK FACTORS FOR *PNEUMOCYSTIS JIROVECII* PNEUMONIA IN KIDNEY TRANSPLANT RECIPIENTS AND APPRAISAL OF STRATEGIES FOR SELECTIVE USE OF CHEMOPROPHYLAXIS

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Conditionally Accepted, Transplant Infectious Diseases

Abstract

Differentiated use of Trimethoprim-Sulfamethoxazole (TMP-SMX) chemoprophylaxis to prevent *Pneumocystis* pneumonia (PCP) in kidney transplant recipients based on risk factor analysis is not a universally adapted strategy and supporting evidence based sources are limited. We performed a large retrospective study to identify risk factors for PCP in kidney transplant recipients and to define parameters for use in clinical prophylaxis guidelines. Fifty consecutive patients with confirmed PCP and two time-matched controls per case were enrolled. Potential risk factors were compared between groups by uni- and multivariate matched analyses. At transplantation, age >55 years and not receiving basiliximab induction therapy predicted development of PCP. In addition, CMV infection (OR 3.0 95%CI 1.2-7.9) and rejection treatment (OR 5.8 95%CI 1.9-18) were independently associated with PCP. Using the variables identified by the multivariate analyses, effects of different hypothetical chemoprophylaxis strategies were systematically evaluated. Exploring different scenarios showed that chemoprophylaxis in the first 6 months for all- and during the first year post-transplantation for patients >55 years or those treated for rejection would result in very low PCP incidence and optimal avoidance of TMP-SMX toxicity. The clinical approach towards application of PCP chemoprophylaxis may be refined accordingly by adopting a both time and risk factor based strategy.

Introduction

Pneumocystis pneumonia (PCP), caused by *Pneumocystis jirovecii* is a recognized cause of morbidity and mortality in kidney- and other solid organ transplant recipients [1, 2]. Recently issued kidney transplantation guidelines recommend the prescription of chemoprophylaxis for at least 3-6 months after transplantation, whereas other sources or expert opinions may advice other time based schedules [3-5]. Trimethoprim-Sulfamethoxazole (TMP-SMX) is the drug of choice and has been proven highly effective in preventing PCP in solid organ transplant recipients [6, 7]. However, although in general the use of PCP chemoprophylaxis after kidney transplantation is a widely accepted practice, a definitive more individualized approach towards the prescription of chemoprophylaxis has not been established [8]. Incentives to abstain from a prophylactic strategy using TMP-SMX include adverse effects e.g. increase in serum creatinine, severe hyperkalemia, gastro-intestinal complaints, Stephen-Johnson's syndrome, interstitial nephritis and interactions with other medication [9-12]. Weighing the incidence and impact of these side effects against the overall morbidity and mortality of PCP among kidney transplant recipients, local renal transplantation program committees or individual physicians may decide when to – or not to – prescribe PCP chemoprophylaxis [13, 14]. Nonetheless, individual cases and 'outbreaks' of PCP that occur in the absence of adequate chemoprophylaxis are reported with some regularity [15-18]. Hence, the need for selective prescription of chemoprophylaxis for patients with an increased risk profile is an important issue to be considered, but few evidence-based sources exist [19]. Two small case-controlled studies with less than 20 cases each, indicated that treatment for graft rejection and Cytomegalovirus virus (CMV) infection are important risk factors [20, 21]. From case series other risk factors were suggested including smoking behavior, use of specific immunosuppressive compounds e.g. Cyclosporine A (CsA) or Tacrolimus (TCR), concurrent Hepatitis C infection, or active tuberculosis [22-26]. In this larger retrospective case-control study the potential risk factors for PCP in kidney transplant recipients are evaluated with the emphasis on the appraisal of previously attributed risks and on the definition of the parameters that could enable future guidelines to contain a differentiated, more individualized approach towards the prescription of PCP chemoprophylaxis in this population.

Methods

Study population

Case patients were identified from the database of the Department of Infectious Diseases of the Leiden University Medical Center, a tertiary care and teaching hospital in the Netherlands with an extensive transplantation program. All consecutive kidney- and combined

kidney-pancreas transplant recipients with clinical signs and symptoms compatible with PCP and with confirmed presence of *P. jirovecii* by direct microscopy (giemsa- and/or silver staining) and/or PCR between January 1983 and July 2008 were included. A standard PCP prophylaxis policy (comprising the first year post transplantation for all patients) was only properly implemented after the end of this period. Control patients were recruited from the transplantation database of the Department of Nephrology. To prevent time period related bias in the analysis, the patients receiving their graft directly before- and after the patient that finally developed PCP were selected as controls. Control patients had to have an at least equal time of immunosuppression as their matched case. Also, they had to be negative for suspicion of PCP throughout their complete follow-up. The use of TMP-SMX (e.g. if prescribed for other indications) or other antibiotics effective as PCP chemoprophylaxis and infection with HIV-1/2 were exclusion criteria for both case- and control patients. If a control patient was excluded, he or she was replaced by the patient transplanted directly before or after this individual. When this method failed, a patient transplanted within a 5 year period was randomly selected as control.

Patient data

Clinical data about mortality, demographic characteristics and the following potential risk factors for development of PCP was collected from the hospital's electronic- and paper patient records and the Nephrology Department's transplantation database: underlying renal and infectious diseases, type of transplant (kidney vs. combined kidney-pancreas), graft origin, immunosuppressive regimen, treatment for graft rejection, smoking behavior and CMV-status. Routine pre-transplantation evaluation included serologic screening for CMV, HIV (from 1985 onward), Hepatitis B and C, and a Mantoux test for the detection of latent or active tuberculosis. Observed time in case patients ranged from transplantation to the day of diagnosis, defined as the day that microbiological evidence of PCP was obtained by broncho-alveolar lavage. For control patients the observed time window ranged from transplantation to the corresponding day of diagnosis in their matched case. Data about the immunosuppressive regimen was obtained at 3 months post-transplantation and at the time of diagnosis (and at corresponding times for control patients). Pre-emptive treatment with the monoclonal antibody basiliximab at the time of transplantation as well as specifics concerning rejection treatment(s) (number, timing and type of medication) within the observed time window was recorded. To investigate the association between CMV infection and PCP, the 3 month period prior to diagnosis of PCP was evaluated. Imperative due to the time period spanned by this study, prove of CMV infection was defined by presence of either a positive pp65 antigen test, CMV seroconversion, rising (4-fold) CMV titers or a positive PCR test for CMV. Surveillance and a low threshold for performing diagnostics in case of suspected CMV infection was the standard of care throughout the period of study. CMV replication as detected by these meth-

ods is referred to as CMV infection throughout the article without discriminating between reactivation and primo infections of CMV. If no test to detect CMV was performed in the time window of interest, this was processed in the analysis as missing data.

Statistical Analysis

Uni- and multivariate (conditional logistic regression for matched data) analyses were performed to assess the association of each of the variables with respect to the risk for development of PCP. Binary variables were incorporated in the multivariate model if the univariate p-value was <0.10 or when incorporation was deemed necessary for logical reasons. Relative risks were approximated by odds ratio's (OR) with 95% confidence intervals (95%CI). A p-value of <0.05 was considered statistically significant. Continuous variables are expressed as medians with interquartile ranges, for binary variables numbers and percentages are given. Using risk factors, identified by the multivariate analysis, the effect of different strategies for prescribing chemoprophylaxis were evaluated (see supplement 1 for details). STATA version 10.0 and SPSS version 17.0 were used to perform all analyses.

Results

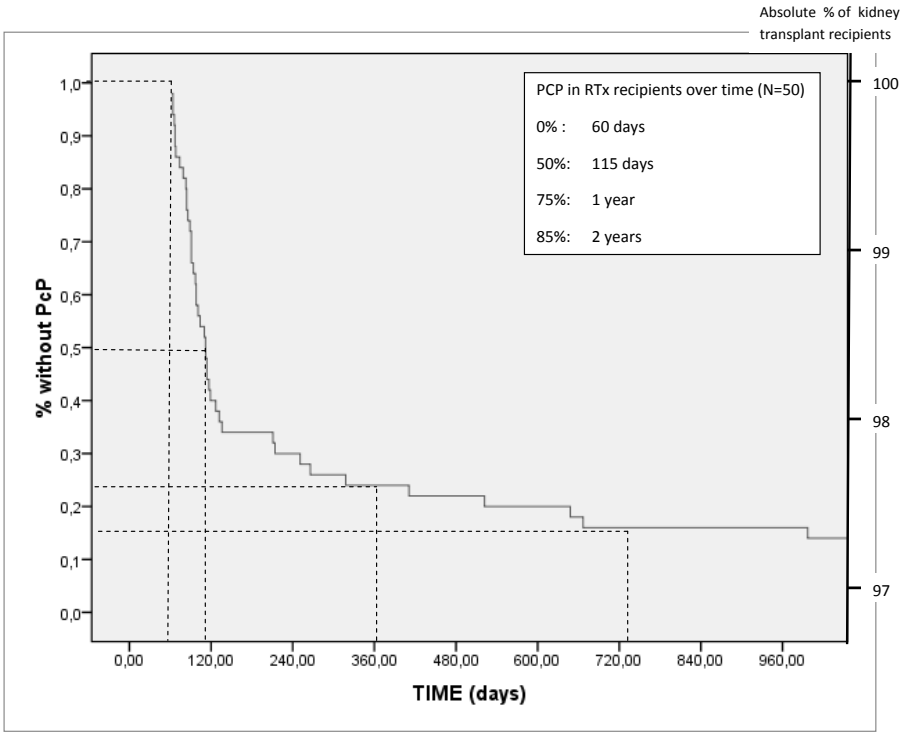
Study population characteristics

Fifty-two kidney transplant recipients with PCP were identified and the overall incidence of PCP was 27 per 1000 transplantation procedures (i.e. 2.7%) in the period of study. None had used SMX-TMP chemoprophylaxis. Two cases were excluded from the analysis due to incomplete data and absence of suitable controls as defined previously. ICU admission and need for mechanical ventilation developed in 4/50 cases and overall 30-day mortality was 6%. Two out of the three patients that died suffered from severe co-morbidity (necrotizing pancreatitis, heart failure). PCP did not occur in the first 60 days post-transplantation despite the absence of prophylaxis; 85% of cases were diagnosed within 24 months after transplantation (figure 1). Some clustering of cases occurred in 2005, but this had no influence on the study outcomes. Uni- and multivariate comparisons of baseline characteristics between case- and control patients are showed in tables 1, 2 and 4A. Due to very low incidences of Hepatitis C infection and active tuberculosis, possible associations could not be established.

Immunosuppressive regimen

At 3 months post transplantation the immunosuppressive regimen generally constituted out of low dose prednisone (5-10 mg/day) in combination with one or two additional compounds.

Figure 1. Development of *Pneumocystis* pneumonia in (study-cohort) kidney transplant recipients over time.



CsA and Mofetyl mycophenolate (MMF) were predominantly used. PCP occurred more frequent when three in stead of two drugs, including prednisone, were used at 3 months post transplantation, but this difference was not statistically significant (OR 1.5 95%CI 0.4-4.9). Use of CsA within the regimen at 3 months showed a trend towards an association with the development of PCP; but these findings were not confirmed at the time of diagnosis nor in the multivariate analysis at baseline (tables 2 and 4A). In case patients, median daily doses of medication at time of diagnosis were 1500 mg (range 1000-2000 mg) for MMF, 75 mg (range 50-100 mg) for Azathioprine (AZA), 3.1 mg/kg (range 1.1-8.4 mg/kg) for CsA and 3 mg (range 2-4 mg) for TCR. Everolimus or Sirolimus were prescribed to less than 5 cases per group. A difference with regard to dosage was found for CsA only, with higher dosages in the group of control patients (median 4.3, range 1.7-12.2 mg/kg; p=0.03).

Treatment for graft rejection and CMV infection

Table 3 summarizes the frequency, number and type of rejection treatments for patients and controls with the corresponding OR's and 95%CI. The standard first treatment was Solumedrol 1000 mg for 3 days. If rejection was steroid resistant, subsequent treatments consisted of anti-thymocyte globulin (ATG) or, if contraindicated, a repeated course of Solumedrol. The

Table 1. Baseline characteristics of kidney transplant recipients with and without development of *Pneumocystis* pneumonia

Variable	Cases (PCP+) No. (%)	Controls (PCP-) No. (%)	OR 95%CI	*p*-value‡
Total No.	**50**	**99**		
Age (years), median (IQR)	57.4 (46-62)	52.1 (42-60)		**0.03**
Age 55+	31 (62)	39 (39)	2.6 (1.3-5.1)	**0.01**
Age 60+	19 (38)	26 (26)	1.9 (0.7-1.7)	0.90
Sex (male)	19 (38)	54 (55)	0.5 (0.2-1.0)	0.05
BMI, median (IQR),	25.7 (23-28)	24.0 (22-26)		0.06
>30 (vs reference category 20-30)	3 (6)	2 (2)	2.6 (0.4-16)	0.29
Type of Transplantation				
Simultaneous Kidney and Pancreas	6 (12)	13 (13)	0.9 (0.3-2.7)	0.86
Kidney	44 (88)	86 (87)	-	-
Donor origin				
Deceased donor	40 (80)	77 (78)	1.1 (0.5-2.7)	0.77
Living donor	10 (20)	22 (22)	-	-
>1 transplantation episode	2 (4)	11 (11)	0.3 (0.1-1.5)	0.15
Primary underlying disease				
ADPKD	8 (16)	20 (20)	0.8 (0.3-1.9)	0.56
Hypertension	11 (22)	19 (19)	1.2 (0.5-2.7)	0.67
DM	14 (28)	23 (23)	1.2 (0.5-2.5)	0.70
IgA nephropathy	2 (4)	5 (5)	0.8 (0.1-4.5)	0.77
Auto-immune diseases[fl]	9 (18)	10 (10)	1.7 (0.7-4.3)	0.23
Recurrent pyelonefritis/other	7 (14)	15 (15)	0.9 (0.3-2.5)	0.87
Unknown cause of renal failure	6 (12)	10 (10)	-	-
Smoking status				
Smoking	7/48 (15)	24/97 (25)	1.1 (0.5-2.6)	0.83
Pre-emptive use of basiliximab at Transplantation	15 (30)	39 (39)	0.3 (0.1-1.1)	0.08

PCP denotes *Pneumocystis* pneumonia; OR: odds ratio; 95%CI: 95% confidence interval; IQR: inter-quartile range; BMI: body mass index; ADPKD: autosomal dominant polycystic kidney disease; DM: diabetes mellitus; ATG: anti-thymocyte globulin. fl: systemic vasculitis e.g. Wegener's granulomatosis, Systemic Lupus Erytematodes etc.; ‡: p-value's determined by univariate matched analysis (binary variables) or Student-t test for continues variables. When a difference was found (p-value cut-off <0.10) variables were included in a binary logistic multivariate model (see table 4).

median duration from transplantation to the first and last rejection treatment given were 16 and 48 days respectively, with 95% of treatments administered within 6 months post transplantation for patients with PCP. These numbers were 15, 36 days and 7 months in the group of control patients. The median interval between the first rejection treatment and PCP was 67 days (IQR 53-81 days). The time to development of PCP inversely correlated with the number of rejection treatments given. If no rejection treatment (either Solumedrol, ATG or both) was prescribed, the median time from transplantation to PCP was 114 days (IQR 90-242 days) and decreased to 104 days (IQR 68-216 days) after one rejection treatment, 98 days (IQR 79-199 days) after two and 87 days (IQR 67-117 days) after ≥3 rejection treatments.

Table 2. Immunosuppressive regimen: maintenance at 3 months and at time of diagnosis in patients with and without development of *Pneumocystis* pneumonia.

Immunosuppressive regimen†	Cases (PCP+) No. (%)	Controls (PCP-) No. (%)	OR (95%CI)	*p*-value
At 3 months post Tx:				
1) CNI + PUR	30 (60)	58 (60)	1.2 (0.4-3.3)	0.79
2) CNI	15 (30)	28 (29)	1.1 (0.4-3.0)	0.87
3) PUR	5 (10)	10 (10)	0.7 (0.2-2.6)	0.60
regimen containing:				
MMF	30 (60)	54 (56)	2.7 (0.6-12)	0.19
CsA	44 (88)	73 (76)	2.9 (1.0-8.3)	0.05
At time of PCP diagnosis:				
1) CNI + PUR	25 (50)	54 (56)	0.7 (0.3-1.8)	0.42
2) CNI	14 (28)	29 (30)	0.9 (0.3-2.4)	0.80
3) PUR	10 (20)	12 (13)	1.7 (0.6-4.7)	0.34
regimen containing:				
MMF	30 (60)	52 (53)	2.4 (0.7-8.5)	0.16
CsA	37 (74)	68 (69)	1.3 (0.6-3.1)	0.46

†: data available for 50 cases and 96 control patients, all used oral prednisolon (median daily dose of 10 mg). PCP denotes *Pneumocystis* pneumonia; OR: odds ratio; 95%CI: 95% confidence interval; MMF: Mofetyl mycophenolate; CsA: cyclosporine A; AZA: Azathioprine; TCR: tacrolimus; CNI: calcineurin inhibitor (i.e. CsA or TCR); PUR: purine synthese inhibitor (i.e MMF or AZA); *p*-values determined by univariate matched analysis (see methods section). Sirolimus and Everolimus were used only incidentally.

Table 3. Treatment for graft rejection as a risk factor for development of *Pneumocystis* pneumonia in kidney transplant recipients.

Variable	Cases (PCP+) No. (%)	Controls (PCP-) No. (%)	OR (95%CI)	*p*-value
No. of patients in the analysis$^{\Sigma}$	42	84		
Total No. of treatments:				
1	6 (14)	10 (12)	2.5 (0.7-8.8)	0.163
2	9 (21)	11 (13)	4.9 (1.3-18.4)	**0.019**
≥3	11 (26)	5 (6)	12.9 (3.0-56.3)	**0.001**
Type of Treatment:				
-Solumedrol only :	8 (19)	12 (14)	1.3 (0.52-3.2)	0.55
-ATG use in at least 1 episode	18 (43)	14 (17)	7.1 (2.0-25)$^{\mu}$	**0.002** $^{\mu}$

∑: the analysis was restricted to all patients with PcP < 2years post transplantation. PCP: *Pneumocystis* pneumonia; OR: odds ratio; 95%CI: 95% confidence interval; ATG: anti-thymocyte globulin; OR: odds ratio; 95%CI: 95% confidence interval; OR, 95%CI and *p*-value as determined by matched approach of the data; μ: corrected for the number of treatments given by use of binary conditional logistic regression with matched approach of data using STATA.

The CMV donor/acceptor serostatus at transplantation was not significantly associated with PCP, even if the donor was CMV positive and the acceptor CMV negative (OR 1.2 95%CI 0.5-2.7). In case patients, CMV infection was more frequently present prior to PCP diagnosis (unadjusted OR 2.7 95%CI 1.2-6.2). In the multivariate analysis both CMV infection (OR 3.0 95%CI 1.2-7.9) and rejection treatment (OR 5.8 95%CI 1.9-18) were independently associated with development of PCP (table 4B).

Table 4. Multivariate analysis of baseline and contemporary risk factors for development of *Pneumocystis* pneumonia in kidney transplant recipients

A) Risk factors for development of PCP at baseline			
Variable	**Adjusted Odds Ratio∫**	**95% Confidence Interval**	***p*-value**
Sex (male)	0.4	0.2-1.0	0.06
Age 55+	2.7	1.3-5.9	**0.01**
CsA containing regimen first 3 months post-transplantation	2.1	0.5-8.5	0.30
TCR containing regimen first 3 months post-transplantation‡	0.2	0.2-2.3	0.22
Basiliximab induction therapy	0.2	0.1-0.9	**0.04**
B) Baseline and contemporary risk factors for development of PCP			
Variable	**Adjusted Odds Ratio∫**	**95% Confidence Interval**	***p*-value**
Sex (male)	0.5	0.2-1.3	0.17
Age 55+ at transplantation	1.9	0.8-4.7	0.18
Treatment for rejection (≥1 vs none) †	5.8	1.9-17.5	**0.002**
CMV infection	3.0	1.2-7.9	**0.02**
Basiliximab induction therapy	0.4	0.1-1.3	0.10

∫ OR's are adjusted for all other variables in the same section of the table. ‡: use of TCR at 3 months post transplantation was added to the analysis to correct for an alternate choice of calcineurine inhibitors with respect to CsA. †: If treatment for rejection was replaced by 'use of ATG' the OR was 4.0 95%CI 1.3-12.5, p=0.015.

Exploring the need for chemoprophylaxis: calculated estimations

The relevant variables from the multivariate analyses were used to describe several strategies for selective use of PCP chemoprophylaxis. The effect of each of the different strategies is described in table 5. Some strategies - depending on the expected incidence - resulted in incidences <1% and a substantially reduced use of chemoprophylaxis. Assuming a 100% effect of TMP-SMX chemoprophylaxis, prescribing PCP chemoprophylaxis between the 2nd and 6th month prevents approximately 80% of cases occurring within 2 years post transplantation and reduces the use of TMP-SMX more than 5-fold as compared to its use for the whole 2-year period. Continuing chemoprophylaxis between the 2nd and 12th month is more effective (prevention of 91% of cases) but results in a higher number of patients (11-50) needed to treat to prevent one case (NNTP). Effective use of TMP-SMX was also predicted if all patients used chemoprophylaxis between the 2nd and 6th month post transplantation and if patients older than 55 years of age and/or patients treated for graft rejection continued this prophylaxis until 1 year post transplantation (prevention of 83-88% of cases, NNTP at incidences 1-5%: 37-7). Adding CMV infection into the strategy rules did not improve the results.

Table 5. Estimated effects of implementation of different selective prophylactic strategies on PCP rate and prescription of TMP-SMX prophylaxis in kidney transplant recipients in the first 2 years post transplantation.

Prophylaxis strategy	Estimated proportion of patients with PCP prevented	Estimated proportion of patients treated	residual frequency | NNTP					
			frequency ∫					
			1.0%		2.5%		5.0%	
#0 No prophylaxis	**0.00**	**0**	1.00	0	2.50	0	5.00	0
#1 All patients 2-24 mo.	**1**	**1**	0.00	100	0.00	40	0.00	20
#2 All patients 2-12 mo.	**0.91**	**0.45**	0.09	50	0.23	21	0.45	11
#3 All patients 2-6 mo.	**0.79**	**0.18**	0.21	24	0.53	10	1.05	6
#4 Age 55+	**0.64**	**0.32**	0.36	51	0.90	21	1.80	11
#5 Treatment for rejection	**0.62**	**0.31**	0.38	51	0.95	21	1.90	11
#6 Age 55+ OR Treatment for rejection	**0.88**	**0.52**	0.12	60	0.30	25	0.60	13
#7 Age 55+ OR Treatment for rejection OR CMV infection	**0.93**	**0.71**	0.07	77	0.18	31	0.35	16
#8 All patients 2-6 mo. & 55+ → 1yr post Tx	**0.83**	**0.27**	0.17	33	0.43	14	0.85	7
#9 All patients 2-6 mo. & 55+ → 2yrs post Tx	**0.86**	**0.44**	0.14	52	0.35	21	0.70	11
#10 All patients 2-6 mo. & RejRx → 1yr post Tx	**0.83**	**0.26**	0.17	32	0.43	13	0.85	7
#11 All patients 2-6 mo. & RejRx → 2yrs post Tx	**0.88**	**0.43**	0.12	50	0.30	20	0.60	11
#12 All patients 2-6 mo. & RejRx or 55+ → 1yr post Tx	**0.88**	**0.32**	0.12	37	0.30	16	0.60	8
#13 All patients 2-6 mo. & RejRx or 55+ → 2yrs post Tx	**0.95**	**0.61**	0.05	65	0.13	27	0.25	14

∫:frequency of PCP as percentage of the total No. of transplantation procedures; Tx denotes transplantation; mo.: months; RejRx: treatment for rejection; yr(s): year(s); 55+: above 55 years of age; NNTP: number needed to treat to prevent one case; → 1 yr post Tx: when TMP-SMX is prescribed until 1 year post transplantation. Grey bars highlight prophylactic strategies that result in a relatively high proportion of cases prevented (>0.8) and a relatively low NNTP.

Discussion

The main findings of this study are that age older than 55 years at the time of transplantation, CMV infection and treatment for rejection were independent risk factors for development of PCP in kidney transplant recipients. No specific immune-suppressive compound was associated with PCP. The vast majority (85%) of PCP cases developed within two years post transplantation. Furthermore, within the first 60 days post transplantation no PCP case was observed. This may be explained by the incubation period as well as by the cumulative suppressive effect on T-cell related immunity. The fact that no cases occurred within these first 2 months post transplantation strongly suggests that the probability that PCP develops in this time window is very low. The observed attributable mortality in this cohort due to PCP was estimated less than 6%. Although higher fatality rates, up to 50%, were previously reported, in more recent publications similar low rates were observed [16, 27]. This may indicate that, due to a multitude of factors e.g. improved post transplantation care and increased awareness among physicians mortality due to PCP in this solid organ transplant population can be diminished.

Analysis of Risk factors for PCP

The study has some limitations due to the retrospective design and the size of the study cohort, although it is to our knowledge the largest study published on this topic up to date. Robust aspects of our study include the time matched approach of both the selection of control patients and the statistical analysis, which prevented skewed results due to changes in e.g. the first choice of immunosuppressive regimen and alterations of diagnostic strategies to diagnose CMV infection over time. Also, next to microbiological ascertainment, the case definition required clinical signs and symptoms compatible with PCP, thereby preventing inclusion of asymptomatic carriers of *Pneumocystis jirovecii* [28].

The occurrence of PCP in renal transplant recipients has previously been linked to the type of immunosuppressive regimen. It was suggested by case series and experimantal studies that CsA in particular increased the risk for PCP in contrast to Azathioprine or MMF [22, 29, 30]. In our study more cases than controles used CsA, but this difference did not reach significance. Noteworthy, CsA was used in a lower dosage in cases as compared to controls, conferring a protective mode of action. This finding remains unexplained by the current knowledge of its effects on either the immune system or *P. jirovecii*.

In concordance with prior observations, we found treatment for graft rejection to be the most important risk factor [20, 21]. A 'dose-dependent' effect was noted, with an OR of more than 10 in patients who received more than 3 rejection treatments compared to patients without rejection. Basiliximab induction therapy at transplantation appeared to be protective which was associated with a decreased need for the use of ATG (data not shown). In the multivariate model to correct for this confounding, basiliximab use as factor that prevented PCP was no longer significant. Presence of CMV infection was independently associated with PCP in uni- and multivariate analyses, suggesting that it is not only an epiphenomenon caused by increased levels of immune suppression. Other clinical studies and in-vitro experiments indicated that CMV may have a direct effect on the cellular immune response [31]. The association we found may be partly ascribed to a decreased immune status due to treatment for rejection, and at least in part to a direct effect of CMV infection on cellular immune responses.

Appraisal of prophylactic strategies for PCP

In modeling the risk for development of PCP, knowledge of exposure rates or risk per patient could increase our understanding of managing the issue of chemoprophylaxis. However, exposure rates for kidney transplant recipients or other patient groups at risk are, for obvious reasons, not available. Although the mode of transmission of *Pneumocystis jirovecii* is heavily debated, increasing consensus exist about asymptomatic carriership in humans as the primary source (in healthy- and immunocompromised individuals) and about interhuman transmission [32-35]. Since it is known from serologic and microbiologic evidence that

more than 80% of infants is exposed within 2 years after birth, it is unlikely that exposure can be avoided long term by kidney transplant recipients [36]. From a clinical practice point of view, the impact of 13 simple selective chemoprophylactic strategies - based on the outcome of this study - was explored at different PCP incidence rates (table 5). Without chemoprophylaxis, the estimated burden of hospital admissions, need for treatment at an ICU and mortality at an incidence <1% is less than 10, 0.8 and 0.5 cases per 1000 transplantation procedures respectively. To maintain these desirable low rates at higher PCP incidences and at the same time avoiding unnecessary use and side effects, several chemoprophylactic strategies seemed feasible. In addition to solely time-based schedules, combining timing with risk factors from the multivariate analysis predicted a more effective use of TMP-SMX and avoidance of unneeded use (60-70%) and subsequent adverse events. Of note, a selective chemoprophylactic strategy may be rolled out only when regular follow-up visits, easy access to high standards of care and awareness of attending physicians is warranted. Next to chemoprophylaxis, other preventive measures, e.g. avoiding contact of patients with PCP with kidney transplant recipients when hospitalized must be considered.

Summary and conclusions

The results of this study provide substantial support for a risk factor based, differentiated approach towards PCP chemoprophylaxis, comprising the first 6 months for all- and for a prolonged period (e.g. during the first year) post transplantation for patients over 55 years of age and those treated for graft rejection. This is partly in line with the recently updated KDIGO guidelines but adds considerably to European guidelines issued in 2002 [5]. As for PCP, chemoprophylaxis may be delayed 4-6 weeks post transplantation. However, depending on local circumstances, other indications (e.g. the risk for Toxoplasmosis) may necessitate the prophylactic use of TMP-SMX in this period [37]. Physicians should also be aware that prolonged prescription of prophylaxis, even more than 2 years post transplantation, sometimes is necessary for those patients at increased risk due to accompanying, conditions (e.g. treatment for lymphoma). Since PCP in kidney transplant recipients remains relatively rare, the safety and effectiveness of the above mentioned strategic approach for managing PCP chemoprophylaxis should be confirmed by long term prospective evaluation of their use in clinical practice.

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Chapter 4 | *Supplement I*

CALCULATION OF THE DIFFERENT PARAMETERS FOR THE EFFECTIVENESS OF SELECTIVE PROPHYLAXIS FOR PNEUMOCYSTIS PNEUMONIA (SUPPLEMENT TO TABLE 5)

Proportion of prevented PCP cases

The effect of a strategy to prevent PCP can be described by the proportion of prevented PCP cases by this strategy (P_{prev}) within a certain time period post transplantation (in this study between the 2nd month and the first 2 years post-transplantation). The (estimated) proportion of prevented cases P_{prev} is calculated by dividing the number of cases that would have prevented (N_{prev}) by a strategy (e.g. strategy X) by the number of cases that would have occurred without this strategy (N_{norm}):

If no strategy is applied $P_{prev} = 0 / N_{norm} = 0$
If a strategy is applied that prevents all cases:
$N_{prev} = N_{norm}$ and thus $P_{prev} = N_{norm} / N_{norm} = 1$
$P_{prev} = N_{prev} / N_{norm}$

In the current described model, the data from the cases (PCP patients) were used to calculate P_{prev}. For example: suppose strategy X was to prescribe prophylaxis from the 2nd month to the first 2 years post-transplantation to all patients older than 55 years. There were 42 patients who experienced a PCP in this period, of whom 27 were older than 55 years at the time of diagnosis. Assuming that the prophylaxis is 100% succesful, this means that $N_{prev} = 27$ and $N_{norm} = 42$ and filling in the formula: $P_{prev} = N_{prev} / N_{norm} = 0.64$. This implies that 64% of cases , could be prevented by strategy X.

Residual frequency

Assume that *I* is the incidence (the frequency of occurrence) of PCP in the general population before applying any stragegy. The incidence (residual frequency) after applying stragey X (I_{res}) can be calculated as:

$I_{res} = (1 - P_{prev}) \times I$

Proportion of patients treated unnecessary

From the data of control patients, the proportion who would have received unneeded prophylaxis can be calculated (Proportion of persons treated, P_{pt}) under a strategy X :

$$P_{pt} = \frac{\text{Number of controls receiving prophylaxis under strategy X}}{\text{total Number of controls}}$$

In this model the control group serves as a representative of the total population of kidney transplant recipients. At low incidences of the disease, the need for correction due to the number of 'would be case' patients receiving prophylaxis in this model can be neglected.

The number needed to treat to prevent one case (NNTP)

To assess the efficacy of a strategy X, we calculated the number needed to treat to prevent one case (NNTP). The NNTP for strategy X is equal to the total number of patients that would receive prophylaxis (i.e. the number of cases that would have been prevented (P_{prev} x *I*) plus the number of controls that would receive prophylaxis ((1-*I*) x P_{pt}), divided by the number of cases that would have been prevented (P_{prev} x *I*):

$$\text{NNTP} = \frac{I \times P_{prev} + (1 - I)P_p}{I \times P_{prev}}$$

Note:

To work with a both useful and practical model, only the most relevant variables from the multivariate analyses (in this study: age >55 years and treatment for rejection, as well as the time windows of interest) should be incorporated in the set of simple prediction rules to explore the effect of different strategies for selective use of prophylaxis as demonstrated in table 5.

Chapter 5

β-D-GLUCAN AND S-ADENOSYLMETHIONINE SERUM LEVELS FOR THE DIAGNOSIS OF *PNEUMOCYSTIS* PNEUMONIA IN HIV-NEGATIVE PATIENTS: A PROSPECTIVE STUDY

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Abstract

Objective: To prospectively assess the diagnostic utility of S-adenosylmethionine (AdoMet) and (1→3)-β-D-glucan (β-D-glucan) serum markers for Pneumocystis pneumonia (PCP) in HIV-negative patients.

Methods: HIV-negative, immunocompromised patients suspected of PCP based on clinical presentation and chest imaging were included. PCP was confirmed or rejected by results of direct microscopy and/or real time PCR on broncho-alveolar lavage (BAL) fluid. Measurement of serum β-D-glucan and AdoMet was performed on serum samples collected at enrollment and during follow-up. Both serum β-D-glucan and AdoMet were assessed for diagnostic accuracy and correlation with clinical and laboratory parameters.

Results: In 31 patients enrolled (21 PCP-positive, 10 PCP-negative), AdoMet levels did not discriminate between patients with and without PCP. Elevated serum β-D-glucan was a reliable indicator for PCP with a sensitivity of 0.90 and specificity of 0.89 at the 60 pg/ml cut-off. In PCP-positive patients β-D-glucan serum levels decreased during treatment and inversely correlated with Pneumocystis PCR cycle threshold values in BAL fluid.

Conclusions: The level of serum β-D-glucan – but not AdoMet – was diagnostic for PCP within the clinical context and may serve as marker for pulmonary fungal load and treatment monitoring.

Introduction

Pneumocystis pneumonia (PCP), caused by Pneumocystis jirovecii is an important cause of morbidity and mortality in patients with human immunodeficiency virus (HIV) infection and other conditions associated with immunosuppression [1]. The diagnosis of PCP is based on microscopy methods (silver, giemsa and immunoflorescent staining) and real-time PCR performed on broncho-alveolar lavage (BAL) samples obtained from patients with a compatible clinical picture [2]. Microscopy techniques are limited by their sensitivity and time demanding procedures. Currently used real time PCR methods to detect P. jirovecii yield high sensitivity but might lack the required specificity by detecting P. jirovecii also in patients who are colonized but do not suffer from PCP [3-6]. Furthermore, the need for both sensitive and specific serum tests for PCP becomes particularly evident when invasive diagnostic procedures cannot be performed due to a patient's clinical condition. Hence, a number of serum markers, including (1→3)-β-D-glucan (β-D-glucan) and S-adenosylmethionine (AdoMet) levels were recently studied for their ability to discriminate between PCP and other pulmonary conditions [7-10].

The polysaccharide β-D-glucan is one of the major components of the cyst wall of P. jirovecii, but is present also in the cell wall of other fungal pathogens e.g. Aspergillus fumigatus and Candida spp. [11, 12]. Its potential as a discriminative marker in serum was first proposed after studies in PCP infected rats as well as in human case series [10, 13]. Watanabe et al. recently demonstrated the high potential of β-D-glucan as a discriminative marker for PCP in a study with HIV infected patients [14]. Yet, prospective data about its use for diagnosing PCP in solid organ transplant recipients and patients with other causes of immunodeficiency is limited. Alternatively, being proposed as a useful biochemical marker in 2003, the measurement of AdoMet levels in serum was recently re-introduced as a promising diagnostic test for PCP in patients infected with HIV, yielding a sensitivity and specificity of >90% [7, 15]. In the cell's metabolism, AdoMet serves as an essential intermediate substance e.g. for methylation reactions and polyamine synthesis. In contrast to almost all other micro-organisms capable of causing disease in humans, Pneumocystis spp. seem to depend on exogenous AdoMet although conflicting data were published [16, 17]. Contrary to high intracellular concentrations, extracellular concentrations are low and may be depleted during PCP [17].

The question has remained whether the observations in HIV-infected individuals with regard to the accuracy of these new serum markers for PCP can be extrapolated to the HIV-negative population. HIV-related PCP and non-HIV related PCP are known to be different in terms of clinical characteristics [18, 19]. Several studies demonstrated that a higher load of P. jirovecii is present in the lungs of patients with HIV as compared to patients with PCP due to other underlying disorders [20, 21]. Despite the lower amount of antigen, the inflammatory response of the immune system appeared to be more severe in HIV-negative immunocompromised individuals with PCP, which is thought to account for the more severe clinical

picture and higher mortality reported in this group [19, 22]. In this study we prospectively assessed whether serum AdoMet, its most direct metabolite adenosylhomocysteine (AdoHcy), the AdoMet/AdoHcy ratio and β-D-glucan, would be reliable indicators for the diagnosis of PCP in HIV-negative immunocompromised individuals. In addition, the correlation of serum AdoMet and β-D-glucan levels with real-time PCR results as well as with other biochemical and clinical parameters were evaluated.

Methods

Patients

In this prospective observational study, consecutive HIV-negative, adult immunocompromised patients suspected of having PCP based on presentation and chest imaging were enrolled during admission in the Leiden University Medical Center, a tertiary care and teaching hospital in The Netherlands. Videobronchoscopy was performed and a segment of an involved lung zone was lavaged using 20 ml aliquots. The diagnosis of PCP was confirmed or rejected by results of direct microscopy methods and/or real-time PCR of the dihydropteroate synthetase (DHPS) gene of P. jirovecii on the BAL fluid [23]. Patients who were thereafter considered PCP negative served as a control group. None of the case- or control patients had other proven invasive fungal infections. Demographical characteristics and data about medical history, symptoms at clinical presentation, treatment and disease outcome were extracted from the medical files. Levels of Lactate dehydrogenase (LDH) and leukocyte count in BAL fluid were acquired from the hospitals' electronic database. PCR cycle threshold values (ct-values) were obtained from the database of the Department of Medical Microbiology. The time of diagnosis was defined as the date when microbiological evidence was first obtained, i.e. the date of the BAL procedure. Data were anonymously noted on case record forms (CRFs) and a database was constructed. The study was approved by the institutional review board of the Leiden University Medical Center and all patients provided written informed consent for participation in the study. Because in our hospital performing a bronchoscopy is part of the standard work-up protocol for immunocompromised patients presenting with pneumonia in which atypical or fungal micro-organisms are suspected to be the cause, informed consent for bronchoscopy was obtained seperately by the lung physician as part of clinical routine.

Sample collection and measurement of S-adenosylmethionine and (1→3)-β-D-glucan

Serum samples were prospectively collected around the time of diagnosis and during one or more time points during the first week of follow-up. Per protocol, blood was drawn for study purposes when venipuncture was performed for other clinical reasons. AdoMet and AdoHcy

were measured by a method adapted from Gellekink et al. [24, 25]. In short, after withdrawal and rapid centrifugation of EDTA-blood for determination of AdoMet and AdoHcy, plasma aliquots were stored at -80C until the time of analysis. AdoMet, AdoHcy and their ratio were determined using liquid chromatography mass spectrometry (LC-MS/MS). After thawing of the non-acidified plasma samples, portions of 10 ul were injected on a 50 x 2.1 mm Atlantis C-18 column (Waters) and eluted in a gradient of methanol in aqueous acetic acid (0.1%). The retention times were 0.6 min (AdoMet) and 1.4 min (AdoHcy). Standards were dissolved in 1 mmol/L HCl; pool sera were AdoMet/AdoHcy depleted by solid phase extraction (SPE) and spiked with the calibrator. Calibration curves for AdoMet and AdoHcy were linear to 500 nmol/l. For the measurement of β-D-glucan, a commercial β-D-glucan assay (Fungitell®, Associates of Cape Cod, Massachusetts, USA) was used. After thawing of samples, the test was performed according to the manufacturers' instructions. All serum tests were performed in batches at the end of the study in a blinded fashion, i.e. the laboratory staff performing the serum tests was unaware of the clinical condition of the patients and outcome of the BAL examinations (which were performed in an other laboratory and by different personnel).

Statistical analysis

Comparisons between groups were performed by use of the Mann-Whitney U test for continuous variables and chi-square and Fishers exact tests for categorical variables. Data were expressed as medians with ranges or interquartile ranges (IQR). Potential correlations between variables were analyzed by use of Kendall's tau-b and Spearman's rho rank correlation tests. Receiver operating characteristic curves were constructed to assess diagnostic accuracy. A p-value of $p<0.05$ was considered significant. All calculations were performed using the SPSS statistical software package for Windows version 17.0.

Results

Study population

Thirty-one consecutive immunocompromised patients suspected of having PCP were enrolled over a 12 month period from March 2005 to March 2006. The majority of patients (65%) were at risk due to receiving a solid organ transplantation (liver transplant: 1, kidney transplant: 19). The baseline characteristics of case and control patients are presented in table 1. None used PCP chemoprophylaxis at presentation. The diagnosis of PCP was ascertained in 21 and rejected in 10 patients. Patients with a solid organ transplantation were overrepresented in the group with PCP (81% versus 30%). No other differences were found between groups. Four out of the 21 patients with PCP were admitted to the ICU and two patients

Table 1. Characteristics of study patients with and without *Pneumocystis* pneumonia

	Patients with PCP (n=21)	Patients without PCP (n=10)	p-value*
Male/female ratio	11/10	8/2	
Age (years), median (IQR)	57 (50-62)	59 (55-70)	0.19
Diagnostic methods (BAL fluid), n (%)			
Microscopy positive	13 (62)	0	
PCR positive	20 (100†)	0	
Underlying condition, n (%)			
Solid organ transplantation	17 (81)	3 (30)	
Hematological malignancy	3 (14)	5 (50)	
Other‡	1 (5)	2 (20)	
Laboratory findings, median (IQR)			
LDH (U/L)	794 (526-1078)	525 (418-599)	0.05
CRP (mg/L)	114 (48-218)	154 (60-217)	0.62
PaO_2 (kPa)	8.3 (7.0-9.2)	8.3 (7.1-9.4)	0.72
Outcome of PCP			
Length of hospitalisation (days), median (IQR)	11 (7-20)	-	
ICU admission, n (%)	4 (19)	-	
30-day mortality, n (%)	2 (10)	-	

Note: PCP: *Pneumocystis* pneumonia; IQR: inter quartile range; LDH: lactate dehydrogenase; CRP: c-reactive protein. ‡:The patient with PCP and 2 patients without PCP were at risk due to Cushing's syndrome or received medication to treat autoimmune disease respectively. †: PCR was not performed in 1 case, but microscopy was positive.*:p-value calculated by Mann Whitney U test.

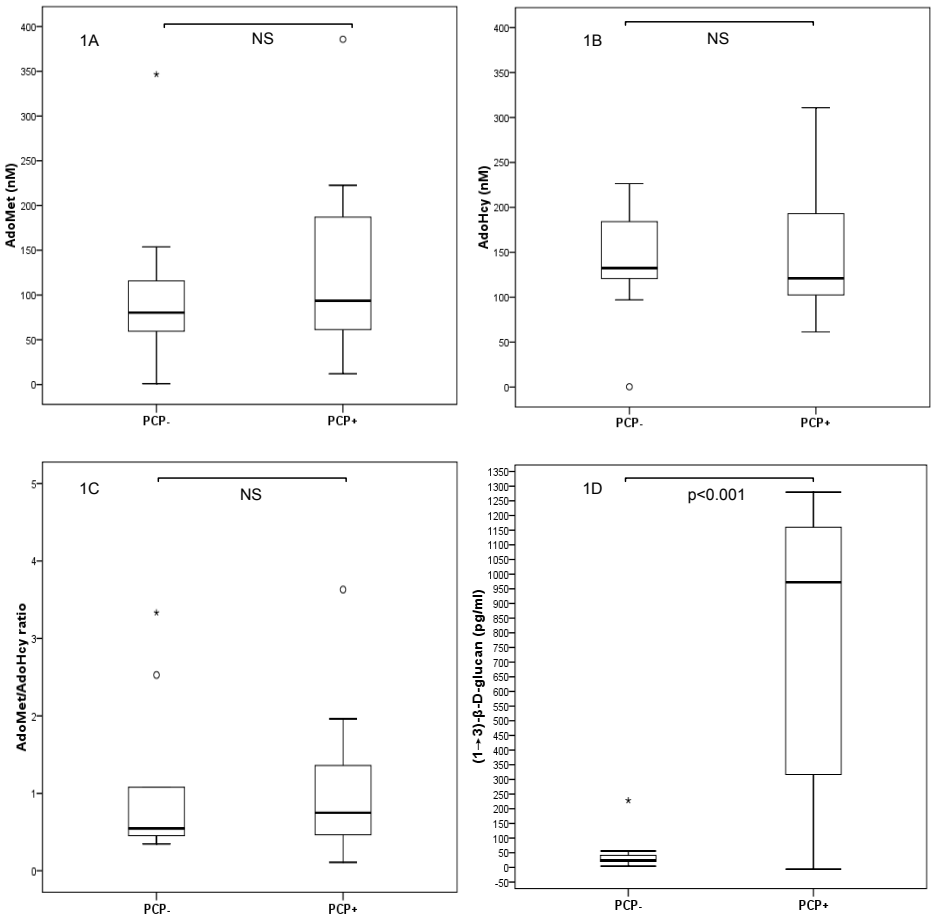
died within the first 30 days following PCP diagnosis. All ascertained PCP cases received Trimethoprim-Sulfamethoxazole (TMP-SM) 2400 mg b.i.d. and steroid treatment according to local guidelines. Among the patients without PCP, 3 had community acquired pneumonia, 5 were diagnosed with lung fibrosis and interstitial pneumonitis related to their underlying disease, 1 patient had a pulmonary infection with Mycobacterium malmoense and 2 patients recovered spontaneously while no definite diagnosis was made. Empirical treatment for PCP was given for a short time (i.e. usually <48 hours) to 6 of these patients while awaiting the results of the BAL examination. None of the control patients developed PCP at a later time point.

Comparisons of serum markers at time of diagnosis

AdoMet

The baseline serum AdoMet concentrations as well as the AdoHcy levels and AdoMet/AdoHcy ratios are shown in figure 1A-C. At initiation of empirical treatment for PCP (which coincided with the timing of the BAL) the median AdoMet level in patients with confirmed PCP was 93.6 nM (IQR 61.1-188.4 nM; range 12.0-385.8 nM) as compared to 80.3 nM (IQR 58.2–125.3

Figure 1. Whisker box-plots of serum levels of AdoMet, AdoHcy, AdoMet/AdoHcy ratio and β-D-glucan in patients with and without *Pneumocystis* pneumonia at time of suspected diagnosis.

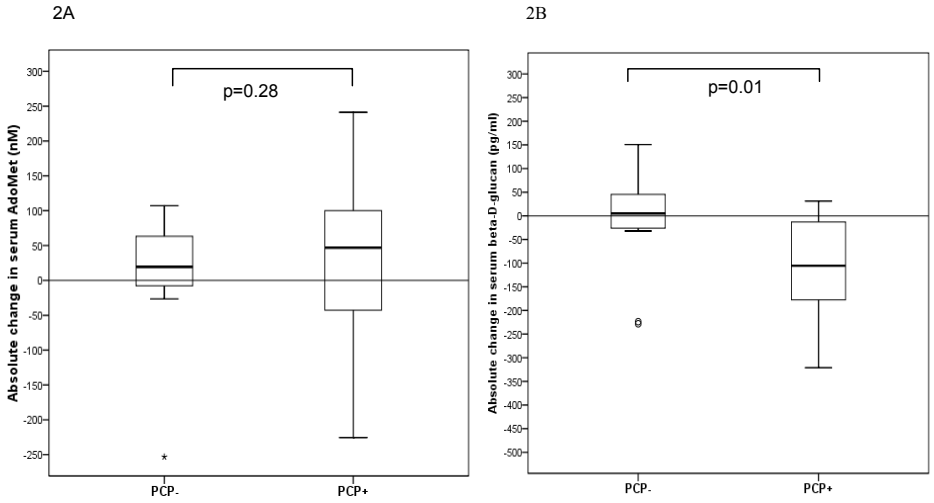


nM; range 1.0-346.6 nM) in the control group (p>0.05; figure 1A). AdoHcy serum levels and AdoMet/AdoHcy ratio were also not significantly different when compared between patients with and without PCP. Follow-up measurement of serum AdoMet levels after a median of 3 days (IQR 2-4 days) of treatment did not show a significant change in patients with PCP as compared to patients without PCP (figure 2A).

β-D-glucan

Serum levels of β-D-glucan were significantly higher in patients with PCP as compared to patients without PCP as depicted in figure 1D. The median serum β-D-glucan level in patients with confirmed PCP was 956.9 pg/ml (IQR 281.6-1171.5 pg/ml; range 0-1279 pg/ml) and 25.3 pg/ml (IQR 14.9-48.4 pg/ml; range 4.5-228.3 pg/ml) in the control group (p<0.001). A second β-D-glucan serum level measured at a median of 3 days (IQR 2-4 days) showed an

Figure 2. Absolute changes in serum AdoMet and β-D-glucan levels between time of diagnosis and follow-up at day 3 in patients without PCP and in patients with PCP on treatment.

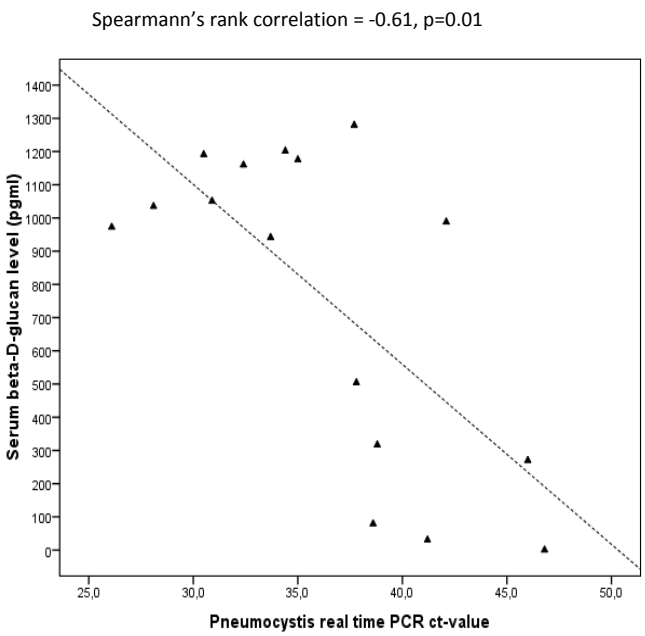


average decrease of 92.9 pg/ml during treatment in patients with PCP compared to a 13.1 pg/ml increase in patients without PCP (figure 2B). At the standard cut-off point of 80 pg/ml, β-D-glucan yielded a sensitivity of 0.86 and specificity of 0.89. When the cut-off point was attenuated to 60 pg/ml, receiver operating characteristic curves showed that the β-D-glucan test performed optimally as an indicator for PCP (AUC 0.89 95%CI 0.75-1.0, p=0.001), with a sensitivity and specificity of 0.90 and 0.89 respectively.

Correlation of β-D-glucan and AdoMet with clinical and laboratory parameters

The median PCR ct-value in of patients with PCP was 34.7 (range 26.1 to 46.8). In this group the β-D-glucan serum levels tended to correlate with the PCR cycle threshold value in BAL fluid (Kendall's tau-b correlation coefficient r=-0.30, p=0.07; Spearman's rho =-0.47, p=0.04). This correlation was more profound when the analysis was repeated for the group with a solid organ transplant only (Kendall's tau-b correlation coefficient r=-0.40, p=0.03; Spearman's rho=-0.61, p=0.01; figure 3). Neither serum β-D-glucan levels nor ct-values of the Pneumocystis PCR (regarded as both dependent on detection of constituents of P. jirovecii) were linked with LDH or CRP in serum at time of BAL. For AdoMet no significant correlations were found between the serum levels and the above variables. Death, ICU admission or length of hospital stay (i.e. time from start of TMP-SM to discharge) was not predicted by β-D-glucan or AdoMet serum levels.

Figure 3. Scatter plot of β-D-glucan levels in serum by *Pneumocystis* real-time PCR cycle threshold value in solid organ transplant recipients with PCP.



Discussion

In this prospective clinical study of immunocompromised patients not infected with HIV, we found that serum β-D-glucan - but not AdoMet - was a reliable indicator for PCP. In addition, we detected a significant correlation between the quantity of P. jirovecii DNA detected in BAL fluid and the serum β-D-glucan level. Furthermore, follow-up levels of β-D-glucan significantly decreased over a relatively short time during treatment. However, the median absolute value measured after a median of 3 days of treatment still remained far above the upper limit of normal, indicating that a clinical response observed at the bedside is of at least equal importance. At present, the kinetics of serum β-D-glucan during PCP are incompletely understood. Previously reported data indicate that, in patients with PCP, the elevated serum β-D-glucan levels only slowly return to normal over a period of several weeks after the start of adequate treatment [26]. Nevertheless, limited data from two small studies investigating the kinetics of serum β-D-glucan suggest that a decreasing β-D-glucan level correlates with the clinical recovery of the patient [27]. Clearly, this aspect of the clinical use of the β-D-glucan assay needs further prospective evaluation.

In HIV infected individuals the clinical relevance of serum β-D-glucan was convincingly addressed recently [14]. However, studies have demonstrated that relatively lower loads of P. jirovecii exist during PCP in the lungs of immunocompromised patients without HIV [20, 21]. In a study performed by Nakamura et al., serum β-D-glucan levels were also significantly

Table 2. Recent studies of β-D-glucan for the diagnosis of Pneumocystis pneumonia in HIV-negative immunocompromised patients.

1st Author (ref)	Year	Journal	N HIV- (N HIV+)‡	Assay used	Design/Main Results
Desmet (8)	2009	J Clin Microbiol	12 (16)	F	Retrospective, case control study: Hematological and HIV-infected patients; serum β-D-glucan yielded a sensitivity of 94% and specificity of 100% at a 100 pg/ml cut-off level.
Del Bono (32)	2009	Clin Vacc Immunol	8 (8)	F	Prospective, case control study: β-D-glucan is a reliable marker for diagnosing PCP. No difference between HIV and non-HIV PCP.
Nakamura (28)	2008	Intern Med	16 (19)	G	Retrospective study: β-D-glucan was a reliable diagnostic marker for PCP. The detection rate of β-D-glucan in non-HIV PCP was lower than in HIV related PCP.
Persat (12)	2008	J Clin Microbiol	4 (16)	F	Part of larger retrospective study, investigating the use of the β-D-glucan assay in a broad spectrum of fungal infection
Marty (33)	2007	Ann Intern Med	13 (3)	F	Case series: β-D-glucan testing may be useful as a noninvasive diagnostic tool for PCP.
Tasaka (9)	2007	Chest	44 (13)	G	Case-control study: HIV-negative patients mainly had collagen-, or hematological diseases. Serum β-D-glucan was a reliable marker for the diagnosis of PCP
Shimizu (34)	2005	Clin Exp Rheum	15 (0)	G	Case series: all patients had connective tissue disease, β-D-glucan testing may be useful as a noninvasive diagnostic tool for PCP.

Note: HIV: human immunodeficiency virus; G: G-test (Seikagaku Corporation, Tokyo, Japan); F: Fungitell test (Associates of Cape Cod, Massachusetts, USA); PCP: Pneumocystis pneumonia; ‡: Due to the mixed population in most β-D-glucan clinical studies, the number of included patients that were HIV-negative (HIV-) and HIV positive (HIV+) are given in column 4.

lower in patients with PCP due to other underlying causes than HIV [28]. These observations question whether serum β-D-glucan levels can be used to reliably aid in the diagnosis of PCP in HIV negative immunocompromised patients. Due to limited clinical data yet available, this issue has not been completely clarified. Table 2 shows an overview of recent English language medical literature on β-D-glucan as a serum marker for PCP in patients without HIV. Although numbers of included patients are small and most of the reported data were obtained retrospectively, these earlier observations concur with the findings of our study. In contrast to the study of Tasaka et al. (n=44), the population of our study is dominated by solid organ transplant recipients [9].

Despite previous investigations claiming AdoMet to be a both highly sensitive and specific marker for PCP in HIV positive patients, this could not be confirmed in our study of a HIV-negative population with PCP. Knowledge about AdoMet as a marker for PCP in the clinical setting is currently driven by one research group only and the method in general is laborious, requires costly, advanced technical equipment and would be hard to sustain in its present form as a test performed in daily hospital routine. High performance liquid chromatography (HPLC) was used in preceding studies investigating the association between serum AdoMet and PCP [7, 29]. In this study we applied the method of stable-isotope dilution liquid chro-

matography–electrospray injection tandem mass spectrometry, a more advanced and now commonly used method for measuring AdoMet and AdoHcy [24, 30, 31]. The mechanism by which AdoMet would be useful as a marker for PCP is its depletion from the serum during infection with P. jirovecii. Since lower pulmonary fungal loads are present in HIV-negative patients with PCP, this may influence the serum AdoMet level in a way that its reliability as a diagnostic test for PCP becomes severely compromised. Moreover, because AdoMet is the product of the human body's own metabolism, other factors e.g. malnourishment, general clinical condition and other variables may easily affect the level of serum AdoMet. For example, it was recently reported that serum folate correlated with AdoMet and that body mass index was the strongest determinant of AdoMet and AdoHcy in multivariable analyses [25].

Summary and Conclusions

Although AdoMet at first appeared a most promising candidate marker, it failed to discriminate between HIV-negative immunocompromised patients with and without PCP. In contrast, serum β-D-glucan levels >60 pg/ml showed high diagnostic accuracy for PCP in HIV-negative immunocompromised patients without other fungal infections. Given the correlation with the PCR cycle threshold value (i.e. a quantative surrogate for presence of P. jirovecii DNA) and significant decrease of the serum β-D-glucan level after treatment initiation, its additional purpose in both research and clinical settings may be that of an indicator of pulmonary load of P. jirovecii. Due to the relatively slow decrease of serum β-D-glucan over time, clinical judgment probably should prevail in estimating the response to treatment. Though, whether in special circumstances (e.g. in case of suspected TMP-SM resistance) the trend of the serum β-D-glucan level may be of additional value, remains to be researched. Due to the 'panfungal' nature of β-D-glucan, its use for the purpose of diagnosing PCP is clearly limited during concurrent infection with other fungal pathogens [12]. The serum β-D-glucan test must therefore not be used as a single test for PCP but interpreted within the clinical context. Thus, a primary suspicion of PCP above other fungal infections (supported by clinical signs and symptoms as well as chest imaging) seems warranted. Under this condition the serum β-D-glucan level may be used as a test with both high sensitivity and specificity for PCP in HIV-negative immunocompromised patients.

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Part II

GENETIC PREDISPOSITION FOR DEVELOPMENT OF INVASIVE ASPERGILLOSIS IN STEM CELL TRANSPLANT RECIPIENTS

Chapter 6

THE Y238X STOP CODON POLYMORPHISM IN THE HUMAN BETA-GLUCAN RECEPTOR DECTIN-1 AND SUSCEPTIBILITY TO INVASIVE ASPERGILLOSIS

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Abstract

Background: Dectin-1 is the major receptor for fungal beta-glucans on myeloid cells. We investigated whether defective Dectin-1 receptor function, due to the early stop codon polymorphism Y238X enhances susceptibility to invasive aspergillosis (IA) in at-risk patients.
Methods: Association of the Dectin-1 Y238X polymorphism with occurrence of IA was evaluated in a cohort of 71 patients who developed IA post hematopoietic stem cell transplantation (HSCT), and in a separate cohort of 21 non-HSCT patients who had IA post-chemotherapy. The control group consisted of 108 patients who had undergone HSCT. Presence of the Y238X polymorphism was linked with the occurrence and clinical course of IA. Functional studies were performed to investigate the consequences of the Y238X Dectin-1 polymorphism.
Results: In HSCT recipients, heterozygosity for the Y238X polymorphism showed a trend towards IA susceptibility (odds ratio 1.79, 95% confidence interval 0.77-4.19, p=0.17). Possession of Y238X polymorphism did not influence the clinical course of IA. The Y238X allele frequency was higher in non-HSCT patients with IA (19.0%) as compared to HSCT patient/donor cohort and the healthy population (range 6.9%-7.7%, $p<0.05$). Functional assays revealed that human peripheral blood mononuclear cells with complete defect in Dectin-1 function due to Y238X responded less efficiently to Aspergillus. However, macrophages showed an adequate response to Aspergillus despite deficient Dectin-1 function.
Conclusions: Dectin-1 Y238X heterozygosity has a moderate influence on susceptibility to IA. This is partly attributable to redundancy inherent in the innate immune system. The Y238X polymorphism may be important in susceptible non-HSCT patients. Larger studies are needed to confirm these findings.

Introduction

Invasive fungal infections (IFI) remain a major cause of morbidity and mortality in immunocompromised patients, of which invasive aspergillosis (IA) is emerging as the most common IFI [1, 2]. Susceptibility and host response to fungal infection is largely determined by the immune status of the host, its ability to recognize the pathogen and to respond appropriately [3]. The mechanism responsible for this recognition is represented by pattern recognition receptors (PRRs) which include the family of Toll-like receptors (TLR) and C-type lectin receptors (CLR) [4]. Dectin-1 is a C-type lectin receptor present on human immune cells e.g. macrophages and monocytes. It recognizes the β-1,3-glucan motif present on the cell walls of Candida and Aspergillus species, and mediates host immune response to these fungal pathogens [5].

Recently, we described a functional single nucleotide polymorphism (SNP) in Dectin-1 (Y238X, rs16910526) leading to an early stop codon which resulted in loss of the last ten amino acids of the carbohydrate-recognition domain of the Dectin-1 receptor. Subsequently, this resulted in diminished expression of the Dectin-1 receptor on immune cells and its inability to bind β-glucan, leading to defective production of proinflammatory cytokines [6]. Clinically, this polymorphism was found to be associated with colonization with Candida spp. in hematopoietic stem cell transplantation (HSCT) recipients [7], as well as recurrent mucocutaneous fungal infection in a Dutch family [6].

Results from in-vitro and murine models have shown that Dectin-1 is pivotal in host defense against Aspergillus infection [8-10]. However, no data is available from human studies to validate these findings. Hence, we aimed to investigate the clinical relevance of the Dectin-1 early stop codon polymorphism for the susceptibility and outcome of IA in a cohort of patients with underlying hematological disorders.

Patients and Methods

Patient population

Ninety-two patients of Dutch-Flemish ancestry with underlying hematological diseases in which IA was diagnosed were enrolled from 3 academic hospitals: Leiden University Medical Center, Radboud University Nijmegen Medical Center, both in the Netherlands, and University Hospitals Leuven, Belgium between May 1996 to July 2009. Of these 92 patients, 71 developed IA following allogenic HSCT while 21 other patients had IA after receiving chemotherapy but without undergoing HSCT. Invasive aspergillosis had been diagnosed as either proven or probable IA as per current European Organization for Research and Treatment of Cancer/Mycology Study Group (EORTC/MSG) criteria [11]. One hundred and eight patients with comparable underlying disorders who underwent HSCT but did not develop IA were

enrolled as controls for the HSCT patients. All HSCT IA patients and the control patients had undergone T cell-depleted allogenic HSCT. The clinical characteristics of the HSCT patients and controls are summarized in Table 1.

Of the 21 non-HSCT patients who developed IA following chemotherapy, 18 patients had acute myeloid leukemia, except for one case each of acute lymphocytic leukemia, multiple myeloma and aplastic anaemia. The median age was 50 years, (interquartile range [IQR] 40-61), and 13 of the 21 subjects (62%) were males. Seventeen of the 21 cases (81%) had probable IA, while proven IA was diagnosed in the remaining 4 patients. Prolonged neutropenia (defined as absolute neutrophil count < 500 cells/mm3 for more than 14 days prior diagnosis of IA) was present in 9/21 cases (43%).

None of the patients or controls in this study received prior mould-active anti-fungal prophylaxis [12, 13]. DNA was obtained from patients following informed consent as required by the ethical committee of each respective institution. For all HSCT cases, DNA was obtained from both recipients and their respective donors prior to transplantation.

Table 1. Patient Demographics and Clinical Characteristics.

Variable	Patients with IA	Patients without IA	p-value
Total No.	**71**	**108**	
Sex ratio m/f	47/24	71/31	0.95
Age (median, IQR)	47 (40-57)	48 (40-56)	0.84
Hematological disease n (%)			
AML	24 (34)	39 (36)	
CML	11 (16)	18 (17)	
ALL	10 (14)	12 (11)	
NHL	9 (13)	14 (13)	
Aplastic anemia	2 (3)	1 (1)	
CLL	3 (4)	7 (7)	
Multiple myeloma	6 (8)	1 (1)	
MDS	6 (8)	16 (15)	
EORTC/MSG 2008 classification			
Proven IA	15	-	
Probable IA	56	-	
Prolonged neutropenia	31/71	40/108	0.46
Site of IA n (%)			
Pulmonary	68 (96)	-	
Extra-pulmonary	3 (4)	-	
GVHD	34/71	58/104†	0.36

The median period of follow-up was 8.4 months (range 0.1-170.7) for patients with invasive aspergillosis and 59.9 months (range 0.4-163.9) for control patients. IA denotes invasive aspergillosis; IQR : interquartile range, HSCT: hematopoietic stem cell transplantation, AML : acute myeloid leukemia, CML : chronic myeloid leukemia, ALL: acute lymphocytic leukemia, NHL : non-Hodgekin's lymphoma, CLL : chronic lymphocytic leukemia, MDS :myelodysplastic syndrome, Prolonged neutropenia was defined as absolute neutrophil count < 500 cells/mm^3 for a period of more than 14 days prior diagnosis of IA, GVHD : graft-versus-host disease. †GVHD data was not available for 4 control patients. P-values were calculated by student-t test for continuous- and Pearson-chi-square test for binary data.

Genotyping for Dectin-1 Y238X Polymorphism

The Y238X SNP (rs16910526) in exon 6 is the only known exonic polymorphism in the Dectin-1 gene in Caucasian populations [7]. Genotyping for the presence of the Y238X polymorphism was performed using the TaqMan SNP assay C_33748481_10 on the 7300 ABI Real-Time polymerase chain reaction system (Applied Biosystems).

Cytokine stimulation assays

Cytokine profiling was performed to ascertain the functional consequence of the Dectin-1 Y238X polymorphism. The isolation of peripheral blood mononuclear cells (PBMC) and differentiation of monocyte-derived macrophages (MDM) from study subjects were performed as previously described [14]. The cells were stimulated with live and heat-killed conidia, as well as with heat-killed hyphae of a well-characterized Aspergillus fumigatus clinical strain, V05-27 [15]. Where indicated, Candida albicans blastoconidia belonging to strain ATCC MYA-3573 (UC820) [16] and particulate β-glucan (courtesy of Dr David Williams, University of Tennessee) were used as control stimuli. The supernatants were collected after 24 h of incubation at 37oC and stored at -20oC until cytokine assay. Interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) concentrations were measured by commercial sandwich ELISA kits (Pelikine Compact, CLB, Amsterdam, Netherlands and R&D Systems respectively) according to manufacturers' instructions.

Flow cytometry

Freshly isolated human PBMC were incubated with 5 μg/ml murine anti-Dectin-1 mAb 259931 (R&D Systems, Minneapolis, MN) or mouse IgG2b isotype control in RPMI 1640 (supplemented with 2% human serum) followed by allophycocyanin-conjugated goat anti-mouse Ab (BD Pharmingen, San Diego, CA). Monocytes were labeled with anti-CD14-PE (Pelicluster, Sanquin, Amsterdam, The Netherlands) and Dectin-1 expression on CD14+ cells was determined by flow cytometry (FACScalibur, BD Biosciences). Detection of Dectin-1 receptor surface expression on MDM was performed as described above. Surface mannose receptor (MR), TLR2 and TLR4 expression was determined using anti-MR-FITC (R&D Systems), anti-TLR2-FITC and anti-TLR4-PE (both from eBioscience, San Diego, CA) in addition to their respective isotype controls.

Statistical Analysis

Genotype frequencies were compared between groups by Fisher's exact- and Pearson-chi-square tests. Odds ratio (OR) and 95% confidence interval (95%CI) were calculated for the

presence (homozygous or heterozygous) or absence (homozygous wild type allele) of the Dectin-1 Y238X polymorphism. Multivariate adjustments for neutropenia and development of graft-versus-host disease (GVHD) were made where appropriate. The influence of the variant Dectin-1 SNP on the clinical course of disease i.e. day from HSCT or start of chemotherapy to the day of diagnosis was assessed by Kaplan-Meier analysis (logrank test). Likewise, associations with presence of the polymorphism and time from IA diagnosis to death were assessed. The cytokine data was presented as mean + standard error of the mean (SEM). Differences in cytokine production were assessed by using Student's t test. A p-value of <0.05 was considered significant. The SPSS version 17.0 statistical software package for Windows was used to perform the calculations.

Results

Dectin-1 Y238X Polymorphism in IA Patients and Controls

Following HSCT, the primary immune cells of the recipient will eventually assume genotype of the donor after successful engraftment. Hence presence of the Dectin-1 Y238X SNP was determined in all patients and HSCT donors. The genotype frequencies of the study cohort were in Hardy Weinberg equilibrium. Thirteen of the 71 patients who developed IA post HSCT (18.3%) and 12 of 108 control patients (11.1%) had the Dectin-1 Y238X SNP. All these individuals were heterozygous for the SNP. Possession of the Y238X polymorphism was only associated with a limited trend towards IA susceptibility and this did not reach statistical significance (odds ratio [OR] 1.79, 95% confidence interval [CI] 0.77-4.19, p=0.17), see Table 2a. Following multivariate adjustment for neutropenia and GVHD, the adjusted OR was 1.70, 95% CI 0.72-4.00, p=0.22. Donor genotype did not influence risk of IA in the recipient. Likewise, simultaneous possession of Y238X in both HSCT donor-recipient pair did not increase susceptibility to IA.

In addition, the Dectin-1 Y238X SNP was found in 7 of the 21 non-HSCT patients (33.3%) who developed IA following immunosuppressive chemotherapy, of which one individual was homozygous for the Y238X polymorphism. Given the limited case patients in this non-HSCT cohort, we opted to compare the allelic frequencies of Dectin-1 Y238X variant against the following patient cohorts/healthy populations: (1) the HSCT patients with and without IA in this study who had similar underlying hematological diseases (2) the healthy HSCT donors (in this study) and (3) healthy population of comparable Dutch ancestry [6] (Table 2b). The allele frequency of the Y238X SNP was significantly elevated in the non-HSCT IA patients (19.0%) as compared to HSCT patients (7.0%, p=0.01), healthy HSCT donors (7.7%, p=0.04) and Dutch population (6.9%, p=0.02).

Table 2a. Incidence of Dectin-1 Y238X variant in HSCT patients/donors between IA cases and controls.

Study Cohort	Cases Dectin-1 Y238X variant present n/N	Controls Dectin-1 Y238X variant present n/N	Univariate OR (95%CI)	p-value
HSCT IA recipients vs control recipients	13/71	12/108	1.79 (0.77-4.19)	0.17
HSCT donors to IA patients vs control donors†	10/68	17/107	0.91 (0.39-2.13)	0.83
Presence of Y238X in both HSCT recipient & donor†	5/68	8/107	0.98 (0.27-2.80)	0.98

† DNA belonging to HSCT donors of 3 IA cases and 1 control case were unavailable for genotyping. IA denotes invasive aspergillosis; HSCT: hematopoietic stem cell transplantation; OR: odds ratio; 95%CI: 95% confidence interval. P-values were obtained by Pearson-chi-square test.

Table 2b. Comparison of allele frequencies of Dectin-1 Y238X variant in susceptible patient cohorts and healthy populations.

Study Cohort:	Non-HSCT patients with IA	HSCT patients (with- and without IA)	Healthy HSCT donors	Corresponding Healthy Population #
Dectin-1 Y238X variant‡	7/21	25/179	27/175	19/138
Allele frequency	19.0%π	7.0%	7.7%	6.9%

IA: invasive aspergillosis. ‡: All individuals were heterozygous for the Y238X Dectin-1 polymorphism except for one individual in the Non-HSCT group who was homozygous. #: Dutch healthy population [6]. π : Frequency of the allele frequency was significantly higher as compared to the all three other populations (Fishers exact test: $p<0.05$), see text for details.

Influence of Dectin-1 Polymorphism on Clinical Course of IA

In addition to its effect on the susceptibility to IA, we assessed whether the presence of the Dectin-1 variant gene might influence clinical course during IA. Kaplan-Meier analysis did not reveal a difference in time-to-development of IA from HSCT between recipients or their donors bearing either the wild-type (WT) or variant allele (Figures 1a &b, p=0.94 and p=0.88 respectively). There was no difference in survival (time to death following diagnosis of IA) consequent to having the WT or variant Dectin-1 allele in both patients and donors (Figures 2 a & b, p-logrank 0.83 and 0.99 respectively).

Figure 1. Time to diagnosis of invasive aspergillosis from start of HSCT conditioning/chemotherapy

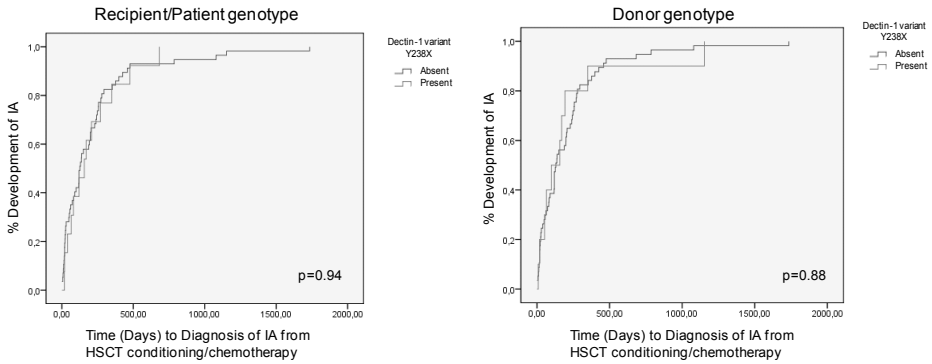
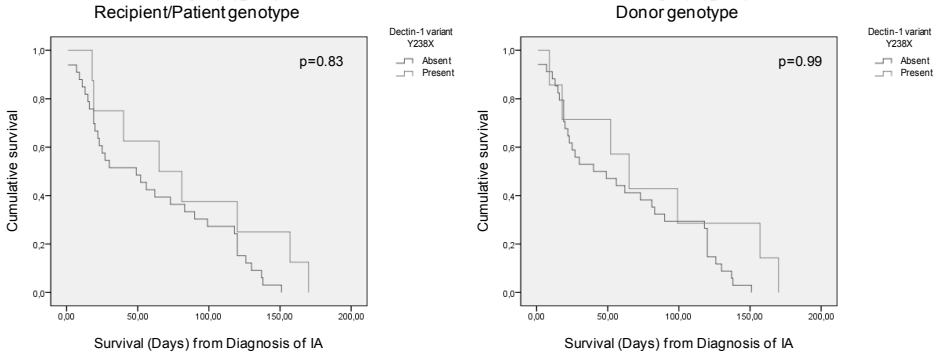


Figure 2. Patient survival following diagnosis of invasive aspergillosis



Functional Consequences of the Dectin-1 Y238X Polymorphism

Functional assays were performed to attempt to find a mechanistic explanation on the limited influence of Dectin-1 on susceptibility to IA. To fully elucidate the phenotypic effects of the Dectin-1 Y238X polymorphism, we had used Dectin-1- deficient PBMC and differentiated MDM from two siblings whom we have characterized as being homozygous for the variant Dectin-1 allele [6], and from healthy control subjects who were WT for the Dectin-1 gene.

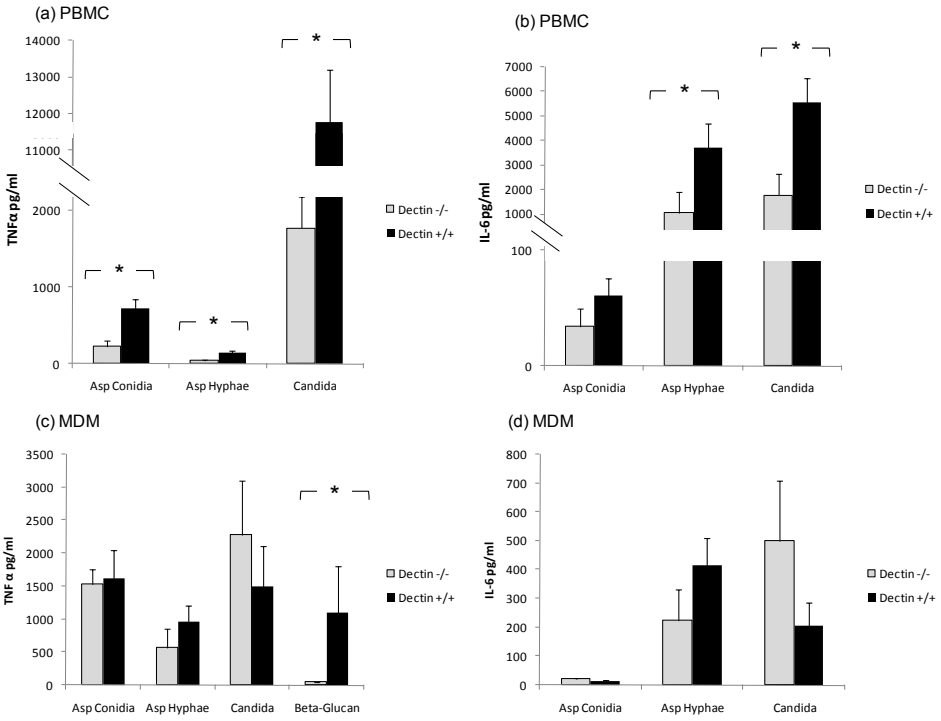
Cytokine stimulation

We assessed the capacity of the immune cells to respond to the various stimuli. In PBMC, homozygosity for Dectin-1 Y238X resulted in marked reduction of proinflammatory TNF-α and IL-6 production in response to heat-killed A. fumigatus hyphae, C. albicans blastoconidia and live A. fumigatus conidia as would have been anticipated given the key role that the Dectin-1 receptor is known to play in recognition of fungal cell wall β-glucan (Figure 3a & b) [7, 9]. In the MDM, however, there were no significant differences in proinflammatory cytokine responses between subjects who were homozygous or WT for Dectin-1. This was apparent for both live A. fumigatus conidia, as well as heat-killed hyphae (Figures 3c & d). As control, stimulation using β-glucan still failed to generate TNF-α response in the Dectin-1-deficient MDM in contrast to MDM containing the wild-type Dectin-1. Despite the intrinsic inability to signal via the Dectin-1 pathway in the Dectin-1-deficient MDM, the demonstrated ability of these cells to still respond to Aspergillus indicated the presence of alternative signaling pathways.

Flow Cytometry

We demonstrated earlier that monocytes from individuals homozygous for Y238X polymorphism had diminished Dectin-1 receptor cell surface expression [7]. We further demonstrate

Figure 3. Functional assays using dectin -/- PBMC and MDM



Legend: Functional assays to assess consequence of the Dectin-1 Y238X polymorphism in response to live *Aspergillus fumigatus* conidia $1x10^7$/ml (Asp Conidia), heat-killed *A. fumigatus* hyphae $1x10^7$/ml (Asp hyphae), *Candida albicans* blastoconidia $1x10^6$/ml (*Candida*) and beta-glucan 20µg/ml. Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) responses of peripheral blood mononuclear cells (PBMC, Fig 3a &b) and monocyte-derived macrophages (MDM, Fig 3c & d) from 2 sibling homozygous for the Y238X polymorphism (Dectin -/-) [6] and 5 Dectin-1 wild-type controls (Dectin +/+) were assessed. Data was from 3 sets of experiments and presented as mean ± standard error of the mean (SEM). * denotes statistical significance $p < 0.05$ as determined by Student's t test.

here that MDM from these individuals also had deficient expression of the Dectin-1 receptor (Figures 4a & c) which is corroborated by the inability to respond to β-glucan as shown above. Besides Dectin-1, other PRRs such as the mannose receptor (MR), TLR2 and TLR4 participate in recognition of fungal ligands [5]. Of note, the MR is a distinct CLR commonly found mainly on macrophages, while TLR2 and TLR4 are ubiquitous on most immune cells including monocytes and macrophages. It is plausible that the host tissue macrophages recognize Aspergillus through these alternative PRRs, especially the MR pathway. This may circumvent deficiency in the Dectin-1 signaling pathway, and account for the normal cytokine production in Dectin-1 deficient MDM. To substantiate this, we showed that Dectin-1 deficient MDM had similar levels of expression of MR as normal cells (Figures 4b & d), and that TLR2 and TLR4 expression was normal (data not shown).

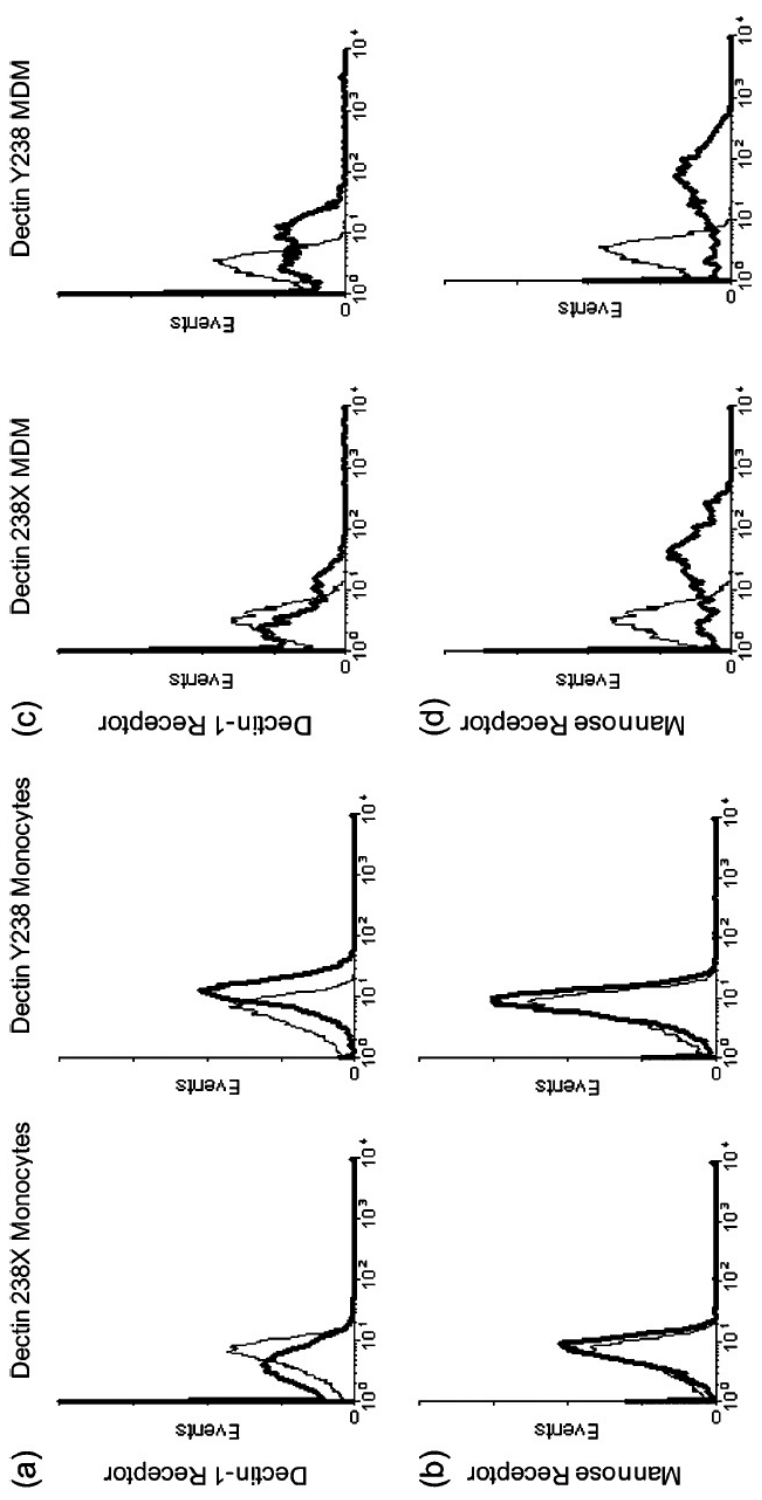


Figure 4. Flow cytometry on Dectin-/- monocytes and MDM

Legend: Representative flow cytometry analysis on surface staining of human Dectin-1 receptor and mannose receptor on Dectin -/- and Dectin +/+ CD14+ monocytes (Fig 4a & b) and MDM (Fig 4c & d). Thin lines represent the respective isotype controls.

Discussion

Though much has been reported on the central role that Dectin-1 plays in host recognition of Aspergillus, this is based largely on findings from experimental murine models. In this study, however, we found that a defective function of Dectin-1 due to a premature stop codon polymorphism may potentially enhance susceptibility to IA in susceptible non-HSCT patients although the effect was moderate in the HSCT cohort and did not significantly alter the clinical course of the disease. In contrast to the observations on Dectin-1 obtained from in-vitro and mice models, the above clinical findings remain significant as they also highlight the system of redundancy inherent in the human innate immune system against invading pathogens like Aspergillus.

Recently, Dectin-1 has been recognized as being a pivotal PRR for the control of fungal infections [17] and specifically for anti-Aspergillus host defense [10]. In-vitro studies demonstrated the involvement of Dectin-1 in both TLR-dependent and TLR-independent anti-fungal responses [14, 18]. The clinical significance of Dectin-1 in mucosal candidiasis was highlighted recently in a study that described how defective Dectin-1 expression and function resulted in recurrent vulvovaginal candidiasis in a family of siblings who were homozygous for the Y238X polymorphism [6], while another study reported an increased incidence of oral and gastrointestinal Candida colonization in HSCT recipients heterozygous for the same variant gene [7].

Despite the in-vitro studies pointing out the importance of Dectin-1 as receptor for fungal β-glucans, the perceived importance of Dectin-1 for invasive mycosis in mice models had been debated. Contrary to findings of Taylor et al that Dectin-1 deficient mice showed increased susceptibility to disseminated candidiasis, another study by Saijo et al did not yield similar corresponding results using an independently developed Dectin-1 -/- mice strain [19, 20]. A later study showed that Dectin-1 had an important role in a murine model of invasive aspergillosis [10]. Nevertheless, it was also interesting to note that a family with siblings who had a complete deficiency of the Dectin-1 function did not report susceptibility to systemic fungal infections [6]. This suggests that although Dectin-1 has an unchallenged role as β-glucan receptor, in the in-vivo situation, alternative recognition pathways can initiate effective anti-fungal responses.

Patient studies remain crucial for the validation of the host defense mechanisms identified in in-vitro and experimental studies. To be at-risk for development of IA, a profoundly immunocompromised status consequent to immune-ablative chemotherapy, HSCT conditioning regimens or chronic corticosteroid treatment is obligate. In this study we found a markedly increased Y238X allele frequency of 19.0% in non-HSCT patients who developed IA post-chemotherapy as compared to other reference populations (range 6.9-7.7%). In concordance to earlier findings from a previous study which reported the Y238X allele frequency to be 6.9%

in the general Dutch population, our analysis of healthy donors to HSCT patients also yielded a comparable allele frequency of 7.7% (p=0.76). One consideration would have been whether the possession of the Y238X polymorphism could remotely be related to acquisition and progression of the underlying hematological disease state resulting in an over-represented allele frequency in the above non-HSCT IA cases. However, this was not the case as we had also determined that in the HSCT patients with similar predisposing hematological disorders, the allele frequency of Y238X was 7.0%; this was comparable to the above healthy populations.

The occurrence of IA in HSCT presents a challenge in studying genetic susceptibility as both donor and recipient genotype will invariably exert their influence on function of the immune cells post transplantation. Even after documented engraftment, it remains unresolved when chimerism is actually achieved at the level of the pulmonary macrophages which form the frontline against the invading Aspergillus. We found that Y238X status in the HSCT recipient was associated with a modest trend towards susceptibility to IA (OR 1.79, 95% CI 0.77-4.19, p=0.17). This was not accentuated following multivariate adjustment (adjusted OR 1.70, 95% CI 0.72-4.00, p=0.22). Also, donor Y238X status was found not to be an attributable factor. Simultaneous presence of the Dectin-1 Y238X variant in both donor-recipient pair did not further confer a dose-dependent effect on susceptibility to IA (Table 2a). Immune recognition and activation at the epithelial level is a key mechanism in host defense against invasive pathogens [21]. Post-HSCT, Dectin-1 expression on epithelial cells and pneumocytes remains as determined by recipient genotype in contrast to immune cells of myeloid origin. Hence this reasonably accounts for our finding that it was the recipient Dectin-1 Y238X status, rather than the donor, which had an influence on susceptibility to IA.

The stronger association observed in the non-HSCT cohort despite the smaller patient numbers may be because patient and treatment profiles are relatively more homogenous. Comparisons incorporating both cohorts, though, may be confounded as there remain inherent differences in treatment regimens (and possibly IA susceptibility) between non-HSCT and HSCT patients [22]. Nevertheless, the increased incidence of the Dectin-1 Y238X variant in non-HSCT IA patients, as well as its association towards IA susceptibility in HSCT recipients, suggest that heterozygosity for the Y238X SNP has a moderate association with acquisition of IA in at-risk patients. Recognizing the potential limitation of our finding - pertaining specifically to the smaller non-HSCT study cohort as well as the methodology employed for the sub-group analysis - further validation of this observation in a larger cohort of non-HSCT IA patients and controls is needed.

Our functional assays using cells isolated from individuals homozygous for the Dectin-1 Y238X polymorphism also shed light on why susceptibility to Aspergillus infection may be limited and clinical course of disease relatively unaltered despite reduced Dectin-1 receptor function. In contrast to the PBMC, Dectin-1 defective MDM had the capability to respond

with normal production of proinflammatory cytokines upon challenge with A. fumigatus. As pulmonary macrophages form the first line of defense against inhaled Aspergillus conidia, our findings highlight the capacity of macrophages to retain their response even with deficient Dectin-1 function probably lies in their capacity to engage alternative PRRs: MR, TLR2 and TLR4. These receptors are known to be involved in immune recognition of Aspergillus and antifungal host defense [23-26]. This ability to retain the capacity to respond to the pathogen in the absence of Dectin-1 underscores the redundancy that is inherent to the human antifungal host defense. On the other hand, the modest susceptibility to aspergillosis in patients bearing the Y238X polymorphism, coupled to the defective monocyte function, suggest an adjuvant yet essential role of infiltrating monocytes for host defense. In contrast to monocytes and macrophages, the main β-glucan receptor on neutrophils is complement receptor 3 [27]. Although neutrophils are important in anti-Aspergillus host defense [28], the Dectin-1 Y238X polymorphism does not affect these cells as neutrophil function was normal in individuals with homozygous Dectin-1 Y238X mutation [6].

Other polymorphisms in genes coding for components of the innate immunity have been recently reported to increase susceptibility to Aspergillus infections: TLR1, TLR4, TLR6 and IL-10 promoter [29-34]. In all of the above studies, like ours, the polymorphism of interest was studied in isolation and not in association with each other. It is tempting to consider that the concomitant presence of two or more of these polymorphisms in a patient may further enhance the risk profile to IA.

In conclusion, in the present study we report that the Dectin-1 Y238X polymorphism was associated with a moderate increase in susceptibility to IA, particularly in non-HSCT immunocompromised patients. Additional studies are needed to validate these findings, yet these data provide novel insight in human host defense during invasive aspergillosis.

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Chapter 7

INFLUENCE OF POLYMORPHISMS IN INNATE IMMUNITY GENES ON SUSCEPTIBILITY TO INVASIVE ASPERGILLOSIS AFTER STEM CELL TRANSPLANTATION

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Abstract

The innate immune system plays a pivotal role in the primary defence against invasive fungal infection. Genetic variation in genes that regulate this response, initiated by pulmonary macrophages, may influence susceptibility to invasive aspergillosis (IA) in patients at risk. We investigated whether common polymorphisms in Toll-like receptor- (TLR) and cytokine genes involved in macrophage regulation contribute to susceptibility to IA. Forty-four allogeneic stem cell transplantation (ASCT) recipients diagnosed with probable- or proven IA according to 2008 EORTC/MSG criteria, were enrolled. The control group consisted of 64 ASCT recipients without IA. The TLR4 1063A>G single nucleotide polymorphism (SNP) was associated with IA when present in donors of ASCT recipients (OR 4.50 95%CI 1.14-17.8, p=0.02). In a multivariate analysis adjusted for occurrence of graft-versus-host-disease and duration of neutropenia, paired presence of the TLR4 1063A>G and IFNG 874T>A or TLR6 745C>T and IFNG 874T>A SNPs showed a trend towards increased susceptibility to IA (p=0.038 and 0.081 respectively). These findings point to the relevant immunological pathway involved in resistance to IA and warrant further study of the effects of TLR and cytokine polymorphisms and their interaction, which may occur on different levels of the complex biological interplay between the immunocompromised host and Aspergillus sp.

Introduction

It is incompletely understood why some hematologic transplant patients develop invasive aspergillosis (IA), a cause of considerable morbidity and mortality, while others remain unaffected [1]. Clinical risk factors for development of invasive fungal infections (IFI), have been identified, but such risks are not absolute [2, 3]. Likely, the host's or, in case of allogeneic stem cell transplantation (ASCT), the donor's genetic signature may influence susceptibility to acquiring manifest IA or at least affect its clinical course. In-vitro- and animal studies indicated that the innate immune system plays a pivotal role in defence against IFI, by pathogen recognition and activation of appropriate host defence mechanisms in pulmonary macrophages [4, 5]. A family of pathogen recognition receptors (PRRs), the Toll-like receptors (TLRs), mediate this process through detection of fungal components and initiation of intracellular signalling pathways that lead to a pro-inflammatory cytokine response [6-10]. Only recently, certain single nucleotide polymorphisms (SNPs) in TLR4 as well as in TLR1 and TLR6 genes were associated with occurrence of IA in ASCT recipients [11-13]. However, the response of the innate immune system relies on a complex network of components which encompasses TLRs as well as molecules of signaling pathways (e.g. MyD88 and NFκB) and subsequently secreted cytokines [14] In this respect, animal studies showed that depletion of IL-12 and IFN-γ delayed pulmonary clearance of A. fumigatus in mice [15]; moreover, a high production of IL-12 and IFN-γ had a protective effect [16]. In humans, little is known about the role of these or other cytokines in the context of innate or acquired anti-fungal defense mechanisms and only scarce data is available to validate the clinical and experimental findings so far. Hence, we aimed to investigate relevance of common genetic polymorphisms, focusing on the TLR-mediated-IL-12-IFN-γ loop to macrophage activation, with regard to susceptibility to development of IA in ASCT recipients.

Methods

Study population

The study cohort consisted of 44 patients with hematological disorders and diagnosed with either proven or probable IA following ASCT according to the revised 2008 European Organization for Research and Treatment of Cancer and Mycosis Study Group (EORTC/MSG) criteria[17]. All patients were treated at the Leiden University Medical Center, a tertiary care and teaching hospital in the Netherlands. Patients were recruited from the database of the Department of Infectious Diseases. Sixty-four patients with comparable hematological disorders but who did not develop IA, were enrolled in the control group. The ethnic background was Caucasian in both groups and all patients had undergone T-cell depleted

Table 1. Clinical characteristics of patients with underlying hematological disease with (cases) or without (controls) invasive aspergillosis developing after allogeneic stem cell transplantation.

Variable	ASCT patients diagnosed with IA	ASCT patients without IA	*p*-value‡
Total No.	**44**	**64**	
Sex ratio male/female	18/26	71/31	0.70
Age (median, IQR)	47 (41-57)	51 (46-58)	0.26
Hematological disease n (%)			
AML	12 (27)	19 (30)	
Multiple myeloma	8 (18)	11 (17)	
CML	8 (18)	5 (8)	
NHL	7 (16)	11 (17)	
ALL	3 (7)	4 (6)	
Aplastic anemia	1 (2)	4 (6)	
CLL	3 (7)	4 (6)	
MDS	2 (5)	4 (6)	
Other	-	2 (3)	
EORTC/MSG 2008 classification			
Proven	5	-	
Probable	39	-	
Site of IA n (%)			
Pulmonary	42 (95)	-	
Extra-pulmonary	2 (5)	-	
Complications n (%)			
Prolonged neutropenia†	15 (34)	29 (45)	0.32
GVHD	25 (57)	21 (32)	0.02

Legenda: IA: invasive aspergillosis; IQR: interquartile range, ASCT: allogeneic stem cell transplantation, AML : acute myeloid leukemia, CML: chronic myeloid leukemia, NHL: non-Hodgekin's lymphoma, ALL: acute lymphocytic leukemia, CLL: chronic lymphocytic leukemia, MDS: myelodysplastic syndrome, †: Prolonged neutropenia defined as absolute neutrophil count <500 cells/mm^3 for a period of more than 14 days. GVHD: graft versus host disease; ‡: p-values were calculated by student-t test for continous- and Fishers exact test for binary data.

ASCT. Demographic and clinical characteristics as well as outcome data were collected from the hospital's electronic database. The duration of neutropenia to the diagnosis of IA was defined as the number of consecutive days from the first day of a granulocyte count <0.5 x10^6 cells/L (determined ±3 times weekly) to the day that microbiological evidence of IA was first obtained. The study was endorsed by the local medical ethics committee. No standard prophylaxis active against Aspergillus sp. was used. Clinical characteristics per group are as summarized in table 1.

Polymorphisms and genotyping

Polymorphisms were considered eligible for study if the SNP was previously reported to be associated with the occurrence of IA and had an expected allele frequency of ≥5% in the population. With regard to the focus of interest as pointed out in the introduction, two SNPs

Table 2. Genetic polymorphisms in the innate immune system of potential influence on susceptibility to invasive aspergillosis.

IL or TLR-gene	SNPdb id	Position nucleotide change	Reported effect	References
IL1B	rs16944	-511C>T	Negatively Influences IL-1β levels. A higher frequency of the *IL1B* -511TT genotype was found in patients with IA as compared to patients without IA.	Wilkinson et al. [23] Sainz et al. [24]
IL10	rs1800872	-592A>C	Promotor SNP, protective effect in conjunction with the -1082 and -819 *IL10* promotor polymorphisms	Seo et al. [25]
IL10	rs1800896	-1082G>A	Promotor SNP, conferring a diminished expression of the *IL10* gene and a subsequent protective effect with respect to IA	Sainz et al. [18]
IL12B	rs41292470	GC>CTCTAA	Promotor SNP, reported influence on response to tuberculosis; association with IA unknown	Sahiratmadja et al. [26]
IFNG	rs2430561	874T>A	Confers dimished production of IFN-γ, resulting in decreased activation of macrophages. Reported to influence cellular response to tuberculosis. Association with IA unknown.	Pravica et al. [27] Rossouw et al. [28]
TLR1	rs5743611	239G>C	Associated with IA in ASCT recipients	Kesh et al. [13]
TLR1	rs4833095	743A>G	Associated with IA in ASCT recipients when present in combination with the *TLR6* 745C>T polymorphism	Kesh et al. [13]
TLR4	rs4986791	1363C>T	Associated with IA when present in donor DNA of ASCT recipients	Bochud et al. [11]
TLR4	rs4986790	1063A>G	Associated with IA in ASCT when present in recipient DNA Associated with IA when present in donor DNA of ASCT recipients	Carvalho et al. [12] Bochud et al. [11]
TLR6	rs5743810	745C>T	Associated with IA in ASCT recipients when present in combination with the *TLR1* 743A>G polymorphism	Kesh et al. [13]

Legenda: IL denotes interleukin; TLR: toll-like receptor; IFN: interferon; ASCT: allogeneic stem cell transplantation; IA: invasive aspergillosis; SNPdb id: Single Nucleotide Polymorphism database identification number.

reported to influence IL-12p40 and IFN-γ production were additionally included (table 2). Blood- or bone marrow samples were used to isolate DNA. Genotyping of polymorphisms was performed by use of a Mass Array platform according to the manufacturer's protocols (Sequenom, San Diego, USA). Multiplex assays were designed using Assay designer software (Sequenom). In brief, after PCR on 2.5 ng of DNA a primer extension reaction was performed to introduce mass-differences between alleles and, after removing salts by adding a resin, ~15 nl of the product was spotted onto a target chip with 384 patches containing matrix. Mass differences were detected using a Bruker Autoflex MALDI-TOF mass spectrometer and genotypes were assigned real-time using Typer 3.1 software (Sequenom). As quality control, 10% of samples were genotyped in duplo; no inconsistencies were observed. Primer sequences are available upon request.

Statistical analysis

Genotype- and allele frequencies were calculated and compared between groups by Pearson-chi-square and Fisher's exact tests. Odds ratio (OR) and 95% confidence interval (95%CI) were calculated for the presence (homozygous or heterozygous) or absence (homozygous wild type allele) of the selected SNPs. All polymorphisms were tested for the Hardy-Weinberg

equilibrium. Due to the possibility that development of IA was influenced by SNPs in the donor DNA, genotype and allele frequencies were also compared between donors of the patients with IA and donors of control patients. Because of expected redundancy and complexity in the pathway to granulocyte and macrophage activation, the relevance of the combined presence of the selected polymorphisms was assessed in a contingency table. The influence of a selected SNP on the course of disease (i.e., duration of neutropenia to day of diagnosis or time from IA diagnosis to death) was assessed by Kaplan-Meier analysis (log rank-test). The SPSS version 17.0 statistical software package for Windows was used for all calculations.

Results

A total of 10 candidate polymorphisms, all acting within the type-1 cytokine loop to macrophage activation, were selected for analysis: five SNPs in three different TLR genes (-1,-4 and -6) and five SNPs in the IL10, IL12B and IFNG genes (table 2). Distribution of genotypes was consistent with the Hardy-Weinberg equilibrium except for the IL12B SNP. The TLR4 1063A>G and TLR4 1363C>T SNPs were in strong linkage disequilibrium, i.e. when the 1063A>G SNP was found, the 1363C>T was almost always also present. No significant difference in genotype or allele frequencies was found between patients with IA and control patients (data not shown). When comparing donor genotype and allele frequencies, the TLR4 1363C>T and TLR4 1063A>G SNPs were more frequently present in donors of patients with IA (table 3).

The donor DNA contained the TLR4 1063A>G SNP in 9 of the 43 case patients (21%) and in 3 (6%) of the 54 control patients successfully genotyped for this polymorphism (OR 4.50 95%CI 1.14-17.8, p=0.02). Following multivariate correction for GVHD and duration of neutropenia the adjusted OR was 3.76 (95% CI 0.90-15.8, p=0.07). In addition, the allele frequency of the IFNG 874T>A polymorphism showed a trend towards association with IA when present in donors of patients with IA (OR 1.60 95%CI 0.91-2.79, p=0.10).

Since our hypothesis was that susceptibility to IA by genetic mutations could be influenced by the interplay of both TLR and cytokine gene mutations, relevance of the combined presence of the selected polymorphisms was assessed in a contingency table (i.e. association of occurrence of IA with the presence of at least one minor allele in both genes in the interaction term). With respect to this analysis no significant associations with IA were found in the comparison of patients with IA versus control patients. However, a similar analysis performed for the genotypes of the donor samples revealed that paired combinations of the TLR4 1063A>G, TLR6 745C>T, or IFNG 874T>A SNPs correlated with occurrence of IA in the recipient (table 4). After multivariate adjustment for GVHD and neutropenia only the association between the TLR4 1063A>G and IFNG 874T>A combination and IA remained statistically significant (p=0.038). When using a forward conditional logistic regression model for assessment of

Table 3. Genotype frequencies of SNPs in *TLR*, *IL10*, *IL12* and *IFNG* genes in the donor DNA of patients who developed invasive aspergillosis after allogeneic stem cell transplantation.

gene	SNP	Distribution of Genotypes (mm/mM/MM) in Donors of ASCT recipients*		Allele frequency of the minor allele		X^2	*p-value*	OR (95%CI)
		cases	*controls*	*cases*	*controls*			
IL1B	-511C>T	2/19/19	5/18/28	0.29	0.28	0.04	0.85	1.07 (0.56-2.04)
IL10	-592A>C	2/15/26	3/19/31	0.22	0.24	0.06	0.81	0.92 (0.47-1.81)
IL10	-1082G>A	5/24/14	13/26/15	0.40	0.48	1.44	0.23	0.70 (0.40-1.25)
IL12B$^{\Sigma}$	GC>CTCTAA	11/21/9	10/39/10	0.52	0.50	0.12	0.73	1.10 (0.63-1.94)
IFNG	874T>A	11/21/11	8/31/22	0.50	0.39	2.71	0.10	1.60 (0.91-2.79)
TLR1	239G>C	1/5/36	0/9/50	0.08	0.08	0.03	0.86	1.10 (0.39-3.08)
TLR1	743A>G	2/16/25	4/20/37	0.23	0.23	0.003	0.96	1.02 (0.53-1.96)
TLR4	1363C>T	1/7/34	0/4/55	**0.11**	**0.03**	**4.37**	**0.04**	**3.42 (1.02-11.5)**
TLR4	1063A>G	2/7/34	0/3/51	**0.13**	**0.03**	**7.17**	**0.01**	**5.13 (1.38-19.0)**
TLR6	745C>T	7/25/10	11/29/19	0.46	0.43	0.20	0.67	1.14 (0.65-2.00)

Legenda: IL denotes interleukin; TLR: toll-like receptor; IFN: interferon; ASCT: allogeneic stem cell transplantation; IA: invasive aspergillosis; X^2: chi-square test value; OR: odds ratio; 95%CI: 95% confidence interval. ∑: distribution of this genotype was not in Hardy-Weinberg equilibrium (p=0.045). *:Due to incidental failing of genotyping the No. of cases and controls are not equal for each SNP; m: minor allele; M:major allele.

Table 4. Final results of the contingency table analysis for the association between the paired presence of TLR and cytokine polymorphisms in donors of ASCT recipients and development of invasive aspergillosis using all 10 polymorphisms.

Paired TLR - or Cytokine SNPs	Unadjusted OR	95% CI	p^{f}	Adjusted* OR	95%CI	*p*
TLR4 1063A>G and *IFNG* 874T>A	10.9	1.29-92.2	0.02	10.2	1.14-91.7	0.038
TLR6 745C>T and *IFNG* 874T>A	2.21	0.99-4.93	0.07	2.02	0.86-4.73	0.105
TLR4 1063A>G and *TLR6* 745C>T	4.11	1.02-15.7	0.05	3.65	0.85-15.7	0.081

f: p-value calculated with Fisher's exact test. IL: interleukin; TLR: toll-like receptor; IFN: interferon. *: Adjusted for presence of GVHD and prolonged neutropenia by binary logistic regression. OR: odds ratio; 95%CI: 95% confidence interval.

strength of the association of individual or paired polymorphisms with IA, incorporating both the single presence of the minor SNP in the TLR4, TLR6 and IFNG genes as well as their paired combinations, showed that the TLR4 1063A>G / IFNG 874T>A combination was most strongly linked with IA (p=0.033).

Kaplan-Meier analysis did not reveal significant differences in time to development of IA between recipients or their donors bearing either only the wild-type or variant allele. There was no difference in survival (time to death following diagnosis of IA) consequent to having one or two minor alleles of the selected SNPs in either recipients or their donors.

Discussion

We found that in this study cohort the TLR4 1063A>G polymorphism was associated with increased susceptibility to IA, when present in the donors DNA of ASCT recipients (alone or in combination with TLR6 745C>T or IFNG 874T>A SNPs). None of the cytokine polymorphisms alone was linked with occurrence of IA. The results of our investigations concur with the study by Bochud et al in which the 1363C>T and 1063A>G polymorphisms in the TLR4 gene were demonstrated to be associated with IA when present in donors of ASCT recipients.[11] In contrast, an increased risk for IA was previously reported for the 1063A>G SNP if present in the recipients DNA but not in the donor DNA [12]. The association between IA and the TLR1 239G>C SNP or between IA and the combination of the TLR1 743A>G and TLR6 745C>T SNPs as reported in a smaller study by Kesh et al. [13], was not confirmed by our data.

The IFNG 874T>A SNP was found to potentially add up to the risk conferred by two of the TLR polymorphisms. Although carriers of this genetic variation produce suboptimal levels of IFN-γ, putting them at increased risk for perhaps manifest tuberculosis, the isolated presence in either donor or recipient did not increase the risk for IA. Remarkably, SNPs that affect the production of IL-10, one of the most important broad-acting negative modulators of the TLR to IL-12 and IFN-γ macrophage-activating pathway, did not influence susceptibility to IA. Absence of IL-10 was demonstrated to cause increased survival of susceptible mice when exposed to Aspergillus fumigatus and in a prospective clinical study a tendency towards protection against IA was detected when the -1082 A/A-genotype was present [18].

As compared to other risk factors, the absolute risk conferred by relevant SNPs in PRR- and cytokine genes is likely to be limited, given the fact that individuals carrying these SNPs do not develop IA unless another immune deficiency is present. Moreover, our data indicate that even in hosts most at risk, the ability to maintain a response to IA is largely unaffected by the studied SNPs, underscoring the already expected redundancy inherent to the human antifungal defense [19]. Likely, specific patterns of genetic polymorphisms rather than a single genetic variation in TLRs or subsequent cytokine pathways that activate macrophages may be associated with IA in patients at risk. The observation of the association between the TLR4 1063A>G plus IFNG 874T>A SNP combination and IA fits such a hypothesis. However, probable associations of IA with conditional combinations of mutations may also attest to the complex immuno-pathogenesis of invasive aspergillosis. As a consequence of neutropenia, the role of key components within the innate immune response (e.g. lung macrophages phagocytosing and eliminating Aspergillus conidia) could be more prominent in the remaining defense against invasive fungal infection and thus facilitate linkage to TLR- in combination with cytokine SNPs. Assuming that the studied SNPs have an effect on the functioning of the innate immune system, different SNPs may also be working at different time points to modulate resistance to IA and eventually constitute the hosts genetic signature of susceptibility.

The study has limitations, e.g. due to a retrospective design and size of the study cohort. However, two studies that explored the role of TLR SNPs and risk for IA included a comparable or even smaller number of patients [12, 13]. Of note, rather than performing a genome-wide analysis, or testing a random collection of immune genes, we chose to investigate the association of IA with pre-set polymorphisms in candidate genes involved in type-1 cytokine loop to macrophage activation. This ameliorates implications with regard to the concept of multiple testing, but by some a significance level of 0.05 may be regarded too liberal. Currently an ongoing discussion about the necessity of p-value adjustment in exploratory epidemiological studies still evolves and with all relevant data reported, final judgment is left to the reader [20, 21]. Furthermore, due to the rapidly evolving research field, producing newly found candidate SNPs like the Dectin-1 Y238X polymorphism, investigations can hardly ever be complete [22].

The overall impact of the reported TLR4 1063A>G and IFNG 874T>A SNPs on the risk of IA should be interpreted with care. Relative risk associations of genetic variations in the case of IA do not stand alone but likely are influenced again by other components in the host's defense. As discussed above, the observations of this study may be accounted for by both a system of redundancy in the innate immune system as well as by the complex biological interaction between the immunocompromised host and the invading fungus. At present, the findings do not extent to the bedside yet, e.g., by providing guidance for individualized prophylaxis or early intervention. However, by further unravelling the interplay between the innate host defence and Aspergillus sp. through experimental and clinical investigations, increased comprehension of the underlying immuno-pathogenetic processes may, in time, translate into insights directly relevant to clinical practice.

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Part III

EXPERIMENTAL MARKERS FOR DETECTION OF FUNGAL INFECTION: SCINTIGRAPHIC IMAGING

Chapter 8

RADIOTRACERS FOR FUNGAL INFECTION IMAGING

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Abstract

Invasive fungal infections are recognized as an important cause of morbidity and mortality in the immunocompromised host. Rapid initiation of adequate antifungal treatment is often hampered by the limitations of current diagnostic methods. This review encompasses the promises and limitations of newer tracers (believed to target the infectious agents), i.e. radiolabeled antimicrobial peptides, antifungals and chitin-specific agents, for fungal infection imaging by scintigraphy. In mice ^{99m}Tc-labeled peptides derived from human ubiquicidin (UBI29-41) and lactoferrin (hLF1-11) distinguished local *Candida albicans* and *Aspergillus fumigatus* infections from sterile inflammatory processes, but not from bacterial infections. Clinical trials showed that ^{99m}Tc-UBI29-41 can distinguish infectious from inflammatory lesions with 80% specificity and 100% sensitivity. ^{99m}Tc-hLF1-11 was able to monitor the antifungal effects of fluconazole on *C. albicans* infections. Moreover, ^{99m}Tc-fluconazole proved to be an excellent tracer for *C. albicans* infections as it did not accumulate in bacterial infections and inflammatory processes. However this tracer poorly detected *A. fumigatus* infections. Furthermore, ^{123}I-chitinase and ^{99m}Tc-HYNIC-CBP21 accumulated in both *C. albicans* and *A. fumigatus* infections in mice at later time points. In conclusion, despite the recent advances in radiolabeled imaging techniques for invasive fungal infections, the search for better tracers for fungal infection imaging should be continued.

Abbreviations: hLF1-11: human lactoferrin peptide corresponding to residues 1-11, UBI29-41: ubiquicidin peptide corresponding to residues 29-41, LPS: lipopolysaccharide, CBP21: chitin-binding protein (21 kDa), HYNIC: hydrazino nicotinamide

Introduction

Positive blood cultures have remained the gold standard for the diagnosis of invasive candidiasis, a condition still associated with high morbidity and mortality [1]. Whether attempted treatment is successful depends on the choice of the antifungal agent and duration of therapy as well as on the elimination of the primary focus [2]. However, patients may have no evident clinical signs or symptoms pointing to the origin of the candidemia or new foci that developed through hematogenous spread. In general, intravascular catheters are most frequently involved as a primary or maintaining focus of candidemia and should then promptly be removed [3, 4]. In surgical patients, infection with *Candida* spp. and subsequent candidemia can be related to the type and site of operation, e.g. intra-abdominal abscesses, mediastinitis, vascular grafts or joint prosthesis. In patients with hematological disorders requiring treatment with bone-marrow transplantation or intensive chemotherapy fungal infections may originate from the gastro-intestinal tract, where the growth of yeasts like *C. albicans* is restricted by the microbial flora, the actions of host's immune defenses, and other local environmental conditions. Disturbance of this balance in severely immunocompromised patients, e.g. loss of the gastro-intestinal mucosal integrity and neutrophil depletion [5], can result in uncontrolled growth of *C. albicans* and lead to invasion of deeper mucosal tissue or dissemination to other organs [6]. Due to an increase in the numbers of these patients it can be anticipated that the absolute number of patients with invasive candidiasis is increasing.

It is often a major challenge for clinicians to determine the primary focus of the infection, the extent of dissemination or whether the surgical site is involved. Important clinical consequences may include extended antifungal treatment, abscess drainage or even removal of the graft or prosthesis [2, 7, 8]. Currently available techniques such as ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) are anatomically oriented. These are highly sensitive and sophisticated, yet lack specificity for infection, especially in early phases, when anatomic structures have not been altered. Furthermore, in presence of distortion of the normal anatomy, i.e. postsurgical changes, scarring, or the presence of implants and/or vascular grafts, the diagnostic role of these techniques is limited. Visualization of fungal infections with fluorine 18- (^{18}F)-fluoro-2-deoxy-D-glucose (^{18}F-FDG) by positron emission tomography (PET) and PET/CT procedures in patients with malignancy has been reported [9]. However, this tracer suffers from serious drawbacks, including its high radiation burden for patients and medical personnel, the requirement of a dedicated imaging equipment (PET scanner), and its short half life (110 min) puts strains on the infrastructure and logistics. No imaging technique that specifically and accurately assesses the presence of *Candida* spp. (or other yeasts/fungi) at different anatomical locations in the human body is currently available to assist clinical decision making. Scintigraphic detection of fungal infections would have the advantage of a whole body image. Thus, nuclear medicine could contribute to fungal infection imaging provided that good tracers are available [10]. The

ideal tracer for fungal infection imaging should fulfill the following criteria: *i*) rapid uptake at sites of fungal infection with little or no accumulation at sites of sterile inflammation and bacterial infections; *ii*) good stability of the labeled complex under physiological conditions; *iii*) preservation of binding activity upon labeling; *iv*) rapid clearance from the circulation with little or no accumulation in unaffected tissues, and *v*) little or no adverse effects, such as toxicity and immunological reactions [11]. Unfortunately, none of the currently available radiopharmaceuticals, e.g., gallium-67-citrate (^{67}Ga), indium-111 (^{111}In), and technetium-99m (^{99m}Tc)-labeled polyclonal human immunoglobulins or monoclonal antibodies, and ^{111}In- or ^{99m}Tc-labeled autologous leukocytes discriminates between infection and sterile inflammation [12-14]. Nevertheless, the visualization of radiolabeled leukocytes has taken a central role in infection imaging during the last decades in clinical practice, resulting in high accuracy for infection diagnosis in selected clinical settings. For obvious reasons, novel tracers for fungal infection imaging are urgently needed.

This review encompasses the current knowledge and future promises as well as the limitations of radiolabeled antimicrobial peptides, antifungals and chitin-binding agents for scintigraphic imaging of invasive fungal infections.

Antimicrobial peptides

Antimicrobial peptides are important components of innate immune system of all living organisms. They are evolutionarily ancient molecules that fend off a wide range of infections. They often contain hydrophobic and cationic amino acids, which are organized in an amphipatic structure. Most antimicrobial peptides interact through their cationic (positively charged) domains with the (negatively charged) surface of microorganisms [15, 16]. Differently from mammalian cells, where the negatively charged lipids are segregated into the inner leaflet facing the cytoplasm, microorganisms expose the negatively charged headgroups, e.g., lipopolysaccharide (LPS), lipoteichoic acids, mannoproteins, to the outer world. Main features of antimicrobial peptides are described in Box 1 and an online catalogue on cationic peptides can be consulted at: *http://www.bbcm.univ.trieste.it/~tossi/antimic.html*.

Difficulties in purifying natural antimicrobial peptides from various sources have prompted the recombinant production of antimicrobial peptides by genetically engeneered bacteria [35] or by peptide synthesis [36, 37]. Sufficient amounts of antimicrobial peptides can be produced under good laboratory practice conditions. The latter is essential for future approval to employ the (radiolabeled) peptides in clinical practice. Peptide synthesis offers the possibility to make chemical variants, such as D-enantiomers, peptides having amino acid substitutions at various positions, peptides in which chelators can be introduced on desired positions to enable labeling via a bifunctional ligand. On the basis of the chemical character-

Box 1.

Key features of antimicrobial peptides

- Antimicrobial peptides usually contain < 50 amino acids with a net positive charge owing to an excess of basic residues, such as lysine and arginine, and ~ 50% hydrophobic amino acids.
- The majority of antimicrobial peptides are derived from larger precursors harboring a signal sequence, whereas other peptides are generated by proteolysis from larger proteins (such as lactoferricin).
- Antimicrobial peptides are part of the chemical barrier against the constant microbial assault found at various anatomical sites, such as epithelia [17].
- The expression of antimicrobial peptides by cells may be constitutive or induced upon contact with microorganisms or their products, like LPS, or pro-inflammatory cytokines.
- Most antimicrobial peptides can affect both planktonic bacteria and those residing in biofilms [18, 19], viruses such as HIV [20], and fungi [21] *in vitro* [22-25] and in laboratory animals [26,28].
- In addition to their antimicrobial actions, antimicrobial peptides such as α- and β-defensins, cathelicidins (LL-37), and lactoferrin-derived peptides, participate at the interface of innate and adaptive immunity by modulating cytokine and chemokine production by a range of cell types, chemoattracting various immune effector cells [29] and mesenchymal stem cells [30], regulating autophagy in conjunction with vitamin D [31], modulating the differentiation of monocytes to macrophages [32] and dendritic cells [33] and stimulating angiogenesis and wound healing [34].

istics (amino acid sequence and secondary structure), and the biological features of domains present in natural antimicrobial peptides/proteins, various potential domains within intact antimicrobial proteins, such as human lactoferrin (hLF) and ubiquicidin (UBI), have been identified [22, 38-40]. Promising candidates for fungal infection detection were selected on the basis of *in vitro* binding studies showing a preferential binding of these peptides to microorganisms over activated human leukocytes, detection of infectious over inflammatory processes in mice, and favorable pharmacokinetics. Further investigations were performed regarding other properties, like immunological adverse effects, toxicology, and antimicrobial activity [41].

To overcome the disadvantage that UBI-derived and lactoferrin-derived peptides (as well as other antimicrobial peptides) exerting their antimicrobial activities and thus destroying their targets for infection imaging, these peptides were used for scintigraphic studies at doses lacking microbicidal activity [42].

Fluconazole

Fluconazole is the most frequently employed among the triazole antifungal agents in treating *Candida* infections in individuals with severe immunodeficiency. It inhibits the ergosterol biosynthesis pathway and, in particular, the cytochrome P450-dependent lanosterol 14α-demethylase, encoded by the *ERG11* gene. Drug-treated cells display a reduction in ergosterol content, the major component of fungal membrane, and accumulation of methylated sterol precursors, such as lanosterol. Such change in sterol composition disturbs membrane function and results in growth inhibition and death of the fungal cells. Indeed, ergosterol contributes to a variety of cellular functions, including fluidity and integrity of the membrane and the proper function of membrane-bound enzymes, such as proteins associated with nutrient transport and chitin synthesis. Ergosterol is also a major component of secretory vesicles in *Saccharomyces cerevisiae*, and has an important role in mitochondrial respiration. Indeed, mutants defective in ergosterol biosynthesis and yeasts treated with azole compounds are induced to a respiratory deficient "petite" status at a high frequency [43]. A further role of ergosterol can be found in mating conditions as demonstrated by mutations in *ERG4*, encoding the enzyme that catalyzes the last step of ergosterol biosynthesis that impair both shmoo formation and cell fusion [44].

Radiolabeling of antimicrobial peptides and fluconazole

Obligatory conditions for adequate radiolabeling include the firm attachment of radionuclide or its incorporation into the antimicrobial peptide/antifungal molecule. Furthermore the labeling yield and the stability of the tracer should be high to allow the visualisation of the infection. The chosen labeling conditions should not affect the binding activity of the peptide/antifungal to the microorganism. The radiolabel is also very important. Technetium-99m is the radiolabel of choice since it is readily available from a molybdenum generator as ^{99m}Tc-pertechnetate, at low cost. In addition, its relatively short half-life (6 hours) results in low radiation burden for the patient. Various methods of labeling peptides with ^{99m}Tc, such as the indirect labeling using the preformed chelate approach or bifunctional chelating agents and the direct labeling method, have been extensively discussed [11, 45]. The radiolabeled tracer obtained with the preformed chelate approach is chemically well-defined as the ^{99m}Tc is bound to the specific chelating moiety before incubation with the peptide, thereby not exposing the compound to harsh labeling conditions. The drawbacks of this method are that it is complex and not well-suited for kit formulation. The use of bifunctional chelating agents incorporated into the peptide molecules, like HYNIC, N_3S, DADT, amongst others, is chemically well-defined. However, introduction of a chelator may alter the lipophilicity of the peptide and thus its pharmacokinetics. Moreover, the large amounts of these chelating agents used to achieve high peptide-chelator conjugation yields require a time-consuming

purification step. The direct labeling method is, however, a simple procedure that has been used to successfully label an array of peptides and antibiotics, e.g. ciprofloxacin [46] and fluconazole [47], while keeping their biological functions intact [48]. This labeling method is performed under reducing conditions at a final pH 5-6 and requires a relatively short labeling time of approximately 10-20 min, resulting in a high labeling yield over 95% [41]. The stability of the ^{99m}Tc-peptide/fluconazole complex in diluted human serum was excellent, and the peptides/anti-infectives retained their antimicrobial activity towards microorganisms in *in vitro* killing assays [41, 42, 49, 50]. In the reaction mixtures no colloids or free radioactive pertechnetate exceeding 5% of the total ^{99m}Tc-activity were observed [41].

In vitro binding studies and scintigraphic detection of infections by ^{99m}Tc-labeled antimicrobial peptides/fluconazole

A first selection of peptides displaying a preferential binding to fungi and bacteria over activated human leukocytes was performed by testing *in vitro* an array of ^{99m}Tc-labeled synthetic peptides derived from natural human antimicrobial peptides, e.g. UBI and hLF [40, 49, 50]. The most promising peptides were ^{99m}Tc-UBI29-41 and ^{99m}Tc-hLF1-11. In an attempt to fulfill all the previously mentioned criteria ^{99m}Tc-labeled fluconazole has also been considered for fungal infection imaging [47]. The results of *in vitro* binding studies of the selected peptides and ^{99m}Tc-fluconazole to *Aspergillus fumigatus*, *Candida albicans*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and activated leukocytes are shown in Table 1.

Following these investigations, ^{99m}Tc-peptides and ^{99m}Tc-fluconazole were injected into mice with a bacterial or fungal infection or sterile inflammatory process in order to study whether they could discriminate infections from sterile inflammatory lesions using scintigraphic techniques. Scintigraphic analysis revealed that these ^{99m}Tc-tracers accumulated at sites of infection, but not inflammation (Figure 1). The results are expressed as the ratio between

Table 1. In vitro binding studies of ^{99m}Tc-compounds to microorganisms and activated leukocytes[a]

Binding per 10^7 cells (% of added radioactivity)					
Compound	*Aspergillus fumigatus*	*Candida albicans*	*Staphylococcus aureus*	*Klebsiella pneumoniae*	Activated leukocytes
^{99m}Tc-hLF 1-11	55±6	26±2	20±4	2±1	10±1
^{99m}Tc-UBI 29-41	52±3	17±1	38±4	11±3	2±0.4
^{99m}Tc-fluconazole	18±2	38±3	3±0.4	6±0.1	13±5
^{99m}Tc-IgG	17±1	17±1	58±2	36±1	8±1

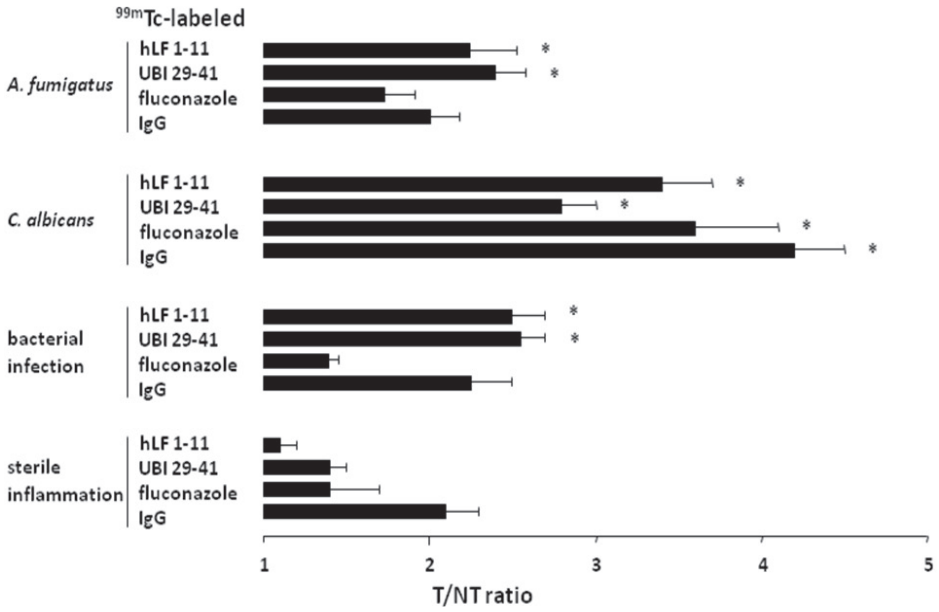
In vitro binding of ^{99m}Tc-labeled compounds to 10^7 CFU of *Aspergillus fumigatus*, *C. albicans*, *S. aureus*, *K. pneumoniae* and activated leukocytes is expressed as the percentage of added radioactivity. Activation of human leukocytes was accomplished after incubation of the cells with a combination of LPS and formyl-Met-Leu-Phe for 1 h at 37°C [Welling 04]. Values are means±SEM of at least eight observations.

[a]From Lupetti *et al.* [Current Drug Targets]

the amount of radioactivity in the infected or inflamed (target) and the non-infected or non-inflamed (non-target) thighs, further referred as to target-to-non-target (T/NT) ratio. ^{99m}Tc-human polyclonal IgG, which accumulates nonspecifically at sites of both infection and inflammation, was used as positive control. The radiolabeled antimicrobial peptides accumulated rapidly (within 30 min) in the target (infected) tissues (1-2% of the injected dose) with little accumulation at sites of sterile inflammation, indicating that fungal and bacterial infections can be distinguished from sterile inflammation by these tracers [47, 50]. After killing the animals, the number of viable microorganisms was assessed in homogenized infected thigh muscles using microbiological techniques. Target-to-non-target ratios for the different tracers are shown in Figure 1.

In contrast to ^{99m}Tc-peptides, which detected both *C. albicans* and bacterial infections in immunocompetent mice, and *A. fumigatus* in immunocompromised mice, ^{99m}Tc-fluconazole accumulated poorly in bacterial and *A. fumigatus* infected thigh muscles [47]. In contrast, a correlation (R^2=0.864, P<0.05; n=12) was found between T/NT ratios at 2 hours after injection of ^{99m}Tc-fluconazole and the number of viable *C. albicans* present in the infected thigh muscles [47]. In previous studies a correlation was seen between the accumulation of ^{99m}Tc-UBI peptides in *C. albicans*-infected thigh muscles in mice and the number of viable yeasts. In

Figure 1. Mean T/NT ratio of infected/inflamed thigh muscles after injection of ^{99m}Tc-labeled compounds.



Legend: Accumulation of ^{99m}Tc-labeled compounds in thigh muscles infected with *A. fumigatus*, *C. albicans*, bacteria (*S. aureus* and *K. pneumoniae*), or inflamed with lipopolysaccharide (LPS). Values represent mean±SEM observed at 30, 60, and 120 min after injection of tracer. * T/NT is significantly higher (P<0.05) than T/NT in mice injected with LPS.

addition, the possibility that ^{99m}Tc-UBI peptides can monitor the efficacy of antifungal therapy against *C. albicans* infection was investigated. The effect of the various concentrations of fluconazole on the accumulation of ^{99m}Tc-UBI29-41 in a fluconazole-sensitive *C. albicans* thigh muscle infection was quantified [51]. The results revealed decreasing amounts of ^{99m}Tc-UBI29-41 at the site of infection with increasing doses of this antifungal agent (R= -0.605; $P< 0.017$). As expected, an inverse correlation (R= -0.788; $P<0.001$) between the number of viable *C. albicans* and the concentration of fluconazole was observed. These data indicate that ^{99m}Tc-UBI29-41 scintigraphy can successfully monitor the efficacy of fluconazole in mice with a disseminated *C. albicans* infection, the lower detection limit being 10^3 colony forming unit (CFU).

Biodistribution

Kinetic studies using intravenously injected ^{99m}Tc-hLF1-11, ^{99m}Tc-UBI29-41, and ^{99m}Tc-fluconazole into *C. albicans* infected mice revealed that the former highly accumulates in the gall bladder and intestines, making it unfavorable for infection detection, whereas ^{99m}Tc-UBI29-41 and ^{99m}Tc-fluconazole are rapidly cleared from the circulation (half-life approximately 30-60 min) via kidneys and urinary tract with low activity in the liver and no intestinal deposits (Table 2). That the activity is mainly cleared through the kidneys is an advantage over hepatobiliary clearance because high activity in the liver and especially in the bowel would have made ^{99m}Tc-tracers less suitable for the imaging of infectious foci in the abdomen (intra-

Table 2. Biodistribution of ^{99m}Tc-labeled compounds in mice infected with *C. albicans.*

Compound	Injected radioactivity (% injected dose)								
	Bladder			*Kidneys*			*Liver*		
Time:	15 min	60 min	240 min	15 min	60 min	240 min	15 min	60 min	240 min
^{99m}Tc-hLF 1-11	12±2	18±3	27±3	15±3	15±2	19±2	24±2	26±2	38±2
^{99m}Tc-UBI 29-41	23±3	32±5	17±3	19±2	22±2	12±2	17±2	14±2	10±1
^{99m}Tc-fluconazole	29±3	34±2	29±7	24±2	22±7	22±4	19±2	10±2	8±7
^{99m}Tc-IgG	17±3	47±2	7±3	14±7	20±2	18±2	17±2	14±2	10±1

Values are means±SD of at least four observations.

From Lupetti *et al.* [02]

abdominal abscesses). In agreement, a previous study which reported on fungal imaging with ^{18}F-fluconazole and PET suffered from rather poor accumulation at sites of infection and high amounts of radioactivity were deposited in the liver [52]. Due to the very different labeling methods ^{18}F-fluconazole is more lipophilic than ^{99m}Tc-fluconazole, and thus is more readily taken up by the liver [53].

Clinical trials with ^{99m}Tc-labeled antimicrobial peptide UBI29-41

Unfortunately, to our knowledge no clinical studies that investigated imaging of fungal infections in patients by radiolabeled tracers have been reported. However, scintigraphic studies in a small series of 18 patients with suspected bacterial bone or soft-tissue infections of the limbs (including prosthesis) showed that ^{99m}Tc-UBI29-41 discriminates between bacterial infections and sterile inflammations in patients and confirmed that this tracer is without adverse effects. Human biodistribution studies showed rapid clearance of ^{99m}Tc-UBI29-41 through the kidneys with gradual accumulation in the urinary bladder in time [54, 55]. Approximately 85% of the injected activity was eliminated by renal clearance 24 hours after injection of the tracer. Accumulation at the site of infection of ^{99m}Tc-UBI29-41 reached the maximum value already from 30 min [55] to 2 hours [56] after intravenous injection. Both in bone and soft tissue infections [55] and in suspected mediastinitis after cardiac surgery [56] the specificity and sensitivity resulted to be around 80% and 100%, respectively. No accumulation of ^{99m}Tc-UBI29-41 was in agreement with negative microbiological results in culture. On the other hand, in most cases, the positive ^{99m}Tc-UBI29-41 scintigraphic images were beneficial for determining the presence of infection and confirmed in microbiological culture. Overall, ^{99m}Tc-UBI29-41 is a highly sensitive and specific agent for localizing bacterial infected foci in various body districts and discriminating them from sterile inflammatory processes.

Newer radioligands

In the attempt to develop new tracers that specifically detect fungal infections, components of fungal cell wall have been considered highly selective targets. Since chitin is a component of fungal cell wall, which is absent in mammalian cells, a radiolabeled marker for chitine, ^{123}I-chitinase was developed in order to bind specifically to fungal cells. The results revealed that this radioiodine labeled enzyme accumulates in *C. albicans* and *A. fumigatus* infections in mice; these infections can be visualized at 24 hours after injection of the tracer and its accumulation correlates with the number of viable fungal cells [57] without visualizing bacterial infections or sterile inflammations. Since radioiodinated peptides are rapidly dehalogenated *in vivo*, ^{123}I was rapidly taken up by thyroid and stomach, resulting in quite disturbed scintigraphic imaging. Furthermore chitinase, which is a protein of ~60 kDa, is not retained in kidneys. To this respect a smaller fragment like the chitin binding domain of this protein

could show better characteristics for *in vivo* imaging, such as rapid uptake, faster blood clearance and therefore faster visualization of fungal infection. Thus, the chitin-binding protein (CBP21; 21kDa) produced by *Serratia marcescens*, which binds chitin with high affinity, has been labeled with ^{99m}Tc via the bifunctional chelating agent HYNIC as a novel radiopharmaceutical for fungal infection imaging [58]. The maximum uptake of ^{99m}Tc-HYNIC-CBP21 was found between 5 and 7 hours postinjection of the tracer. T/NT ratios for *A. fumigatus* were significantly higher than T/NT ratios for *C. albicans*, maybe related to a different chitin percentage in their cell wall or difference in the accessibility of the chitin. Similar results were found in *in vitro* binding studies. T/NT ratios for fungal infection were higher than T/NT ratios for bacterial infections and sterile inflammation between 5 and 7 hours postinjection but not earlier. Clearance was via kidneys and urinary bladder but the uptake in stomach was high up till 7 hours after injection of the tracer. Since it is generally understood that stomach uptake is due to ^{99m}Tc-O_4^- and the tracer was > 97% pure, these data point to instability of the tracer *in vivo* [58]. Potential limitations of these newer tracers are the following. First, since chitin is an abundant substance in nature, chitinase or a chitin binding protein might be non specific for fungal cells. In addition, Guan *et al.* have been recently demonstrated that the chitinase BjCHI1, identified to possess two chitin-binding domains, inhibits not only the growth of fungi but also the growth of gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*) more effectively than Gram-positive bacteria (*Micrococcus luteus*, *Bacillus megaterium*) through its agglutination activity [59]. Therefore, depending on the bacteria causing the infection, ^{123}I-chitinase as well as ^{99m}Tc-HYNIC-CBP21 could result in non specific tracers for fungal infections. Second, it has been recently demonstrated that acidic mammalian chitinase is produced by lung epithelial cells, macrophages and eosinophils at sites of Th2 inflammation [60], being expressed in an exaggerated fashion in epithelial and inflammatory cells in tissues from patients with moderate-severe asthma. Furthermore, the serum level of the chitinase-like protein YKL-40, that was elevated in patients with asthma, was correlated with severity, thickening of the subepithelial basement membrane, and pulmonary function [61]. Therefore, injection of these tracers in humans could have important immunological side effects.

Conclusions

Newer tracers, i.e. radiolabeled antimicrobial peptides, fluconazole and agents targeting chitin, may be useful for imaging of fungal infections. The main limitation of radiolabeled antimicrobial peptides - that discriminate between infections and sterile inflammatory processes - is that they can not distinguish fungal infections from bacterial infections. However, radiolabeled antimicrobial peptides were successful in monitoring antifungal therapy in *C. albicans*-infected mice. Moreover, radiolabeled fluconazole distinguished *C. albicans*

infections from bacterial infections/sterile inflammatory processes, but failed to image *A. fumigatus* infections. In addition, ^{99m}Tc-UBI29-41 and ^{99m}Tc-fluconazole are eliminated from the circulation mainly via the urinary tract, thus showing a favorable pharmacokinetics. Finally, radiolabeled agents that target chitin may be an interesting option for fungal infection imaging at later time points. Further studies exploring novel radioligands able to distinguish invasive fungal infections from bacterial infections as well as from sterile inflammatory processes are needed.

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Chapter 9

GENERAL DISCUSSION AND SUMMARY

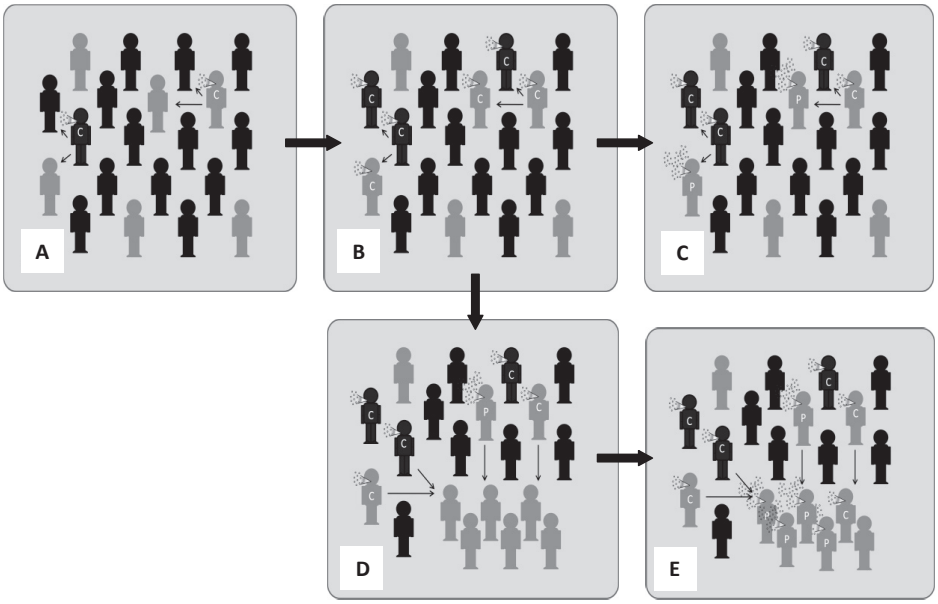
General Discussion and Summary

Pneumocystis pneumonia (PCP) and invasive aspergillosis (IA) are the most prevalent opportunistic pulmonary fungal infections occurring post transplantation. About both pathogens, but in particular about the in-vitro unculturable *P. jirovecii*, a high level of uncertainty exists with respect to transmission patterns and the dynamics of exposure. In the near future - for PCP as well as for invasive aspergillosis - the detailed assessment of the clinical risk factors (including the genetic make-up of the host) is of major importance and the single path to selective prevention strategies. Where exposure is inevitable and prevention strategies fail, the next line of defense is formed by the application of sensitive and specific non-invasive tests to allow early diagnosis and/or monitoring for disease. With regard to the above, the observations and results of the studies described in the **Chapters 2 through 8** are summarized and concisely discussed in the sections below.

Transmission of *Pneumocystis jirovecii* and identification of factors that cause the occurrence of an outbreak of *Pneumocystis* pneumonia in kidney – and possibly other – transplant populations.

Triggered by a sudden rise in the incidence of PCP among kidney transplant recipients in our institution, we set out to investigate the underlying cause. The outbreak investigation and the systematic literature review described in **Chapters 2 and 3** were performed to elucidate the origin of the outbreak as well as the mode of transmission of *P. jirovecii*. From the presented epidemiological data *alone* the presence of an environmental source could neither be confirmed nor excluded. The same was true for possible interhuman transmission. For example: the communal presence of patients in the outpatient department might imply that they acquired PCP through interhuman transmission just as easily as it can indicate that they were infected by a local environmental source. The genotyping shows that patient-to-patient transmission cannot be excluded, but still allows the possibility of a single strain that infects patients from its environmental niche. Also, the statistical approach to the outbreak data described in **chapter 2** yields ambiguous results. The analysis of outpatient visits of PCP patients and frequency of encounters with patients who later developed PCP showed the strongest association with the number of times that a patient visited the outpatient department (Cox conditional regression model). Since statistical models represent an abstraction of reality, it is uncertain whether these calculations can reliably assess the likelihood of either interhuman transmission or a common environmental source. However, in the greater evolutionary context of the commensal relation that likely exists between *P. jirovecii* and humans, there is only one preferred model of transmission of *P. jirovecii* and development of PCP as pointed out in the following paragraph [1, 2].

Figure 1. Model of transmission of *Pneumocystis jirovecii* within the human population and occurrence of *Pneumocystis* pneumonia outbreaks in populations at risk.



The discovery of the linkage of each species of *Pneumocystis* to a specific mammalian host and the phenomenon of common asymptomatic carriage in the airways of both healthy and immunocompromised hosts strongly attest to the hypothesis that the human population forms the primary – if not the only – source [3, 4]. With the human population identified as a reservoir, *P. jirovecii* circulates among both immunocompromised and healthy individuals by interhuman airborne transmission (panel 1A-B). Immunocompromised individuals, upon contracting *P. jirovecii*, may, or may not, develop symptomatic disease (i.e., PCP). This depends, among other factors, on the specific state of their cell mediated immunity and yet unknown virulence factors of *P. jirovecii* (panel 1C). In contrast to the emergence of solitary cases of PCP in kidney transplant recipients explained by this model, a set of specific additional factors (e.g., crowding) leading to increased exposure and/or enhanced susceptibility probably needs to co-exist in order to give rise to a PCP outbreak among an immunocompromised population (panels 1D-E). Exposure is probably increased through frequent contact or crowding of individuals at increased risk for either carriage of *P. jirovecii* or development of PCP, as was convincingly demonstrated to be the case in a large number of outbreak studies included in the systematic review (**chapter 3**).

Clinical risk factors and approach to chemoprophylaxis for *Pneumocystis* Pneumonia in HIV-negative immunocompromised hosts.

Individual cases and outbreaks of PCP that occur in the absence of adequate chemoprophylaxis are still regularly reported in the medical literature [5, 6]. Transplant physicians sometimes abstain from a prescribing chemoprophylaxis for a variety of reasons including anticipation on adverse effects e.g. increase in serum creatinine, hyperkalemia, Stephen-Johnson's syndrome, interstitial nephritis and interactions with other medication [7-9]. The pros and cons of this approach were heavily debated by experts in the field [10, 11]. In the absence of well-designed trials, kidney transplant guidelines recommend the prescription of chemoprophylaxis for 'at least 3-6 months after transplantation' [12]. The alternative of a selective, i.e. more individualized approach towards the prescription was not yet explored. In **chapter 4** we demonstrate by multivariate analysis that age older than 55 years at the time of transplantation, CMV infection and treatment for rejection were the main independent risk factors for development of PCP in kidney transplant recipients. When these variables are incorporated in a hypothetical risk factor and time dependent prophylaxis strategy, the expected effects for the Leiden kidney transplantation cohort were estimated. The model showed that by use of several selective strategies the use of chemoprophylaxis within the first two years post transplantation could be decreased by 60 to 70% while maintaining the PCP incidence at <1.0 %.

The implementation of the results of these findings is complicated by some limitations. First, PCP remains a relatively rare diagnosis and validation of a chemoprophylactic strategy may take years. In addition, the population characteristics (e.g., age or the frequency of rejection treatment) may change over time. Furthermore there is an increased 'physician failure hazard' since it has to be specifically determined whether a patient needs, or does not need a prescription for PCP chemoprophylaxis. A certain strategy may work for years but will finally become redundant or inappropriate due to changes in the standard immunosuppressive regimen, new treatments for treatment for rejection or development of new virulence factors by *P. jirovecii*. At present however, our study provides substantial support for a risk-factor-based, differentiated approach towards PCP chemoprophylaxis, comprising the first 6 months for all- and for the first year post transplantation limited to patients over 55 years of age and those treated for graft rejection. This recommendation adds to current European and other kidney transplantation guidelines [13]. It should be noted that prolonged prescription of prophylaxis, even over years post transplantation, may sometimes be necessary for those patients at increased risk due to accompanying conditions [14].

Biological determinants of invasive aspergillosis and the potential influence of genetic polymorphisms in the innate immune system on host susceptibility to disease.

To be at risk for development of IA, a profoundly immunocompromised state, such as observed after immune-ablative chemotherapy, allogeneic stem cell transplantation (ASCT) conditioning regimens or chronic corticosteroid treatment, is needed. Study of the influence of host genetics on susceptibility to IA in ASCT recipients represents a challenge while both donor and recipient genotype will invariably exert their influence on function of the immune cells post transplantation. It is uncertain when chimerism is achieved at the level of the pulmonary macrophages, which cells constitute the frontline in the immune response to invasive fungal infection (IFI). For the activation of this immune response, recognition of fungal antigens by pattern recognition receptors (PRRs), which include C-type lectin receptors like the Dectin-1 receptor, is pivotal [4]. Recently, a functional single nucleotide polymorphism in this receptor (Y238X), which resulted in diminished expression of the Dectin-1 receptor on immune cells, was described [6].

In the study presented in **chapter 6** we found that Y238X status of the ASCT recipient was associated with a modest trend towards susceptibility to IA. After multivariate adjustment, the Y238X status was no longer significant as a risk factor for IA. The increased Y238X allele frequency of 19.0% in non-ASCT recipients with IA (as compared to other reference populations: range 6.9-7.7%) suggest that heterozygosity for the Y238X SNP potentially has a moderate association with acquisition of IA in patients at-risk in some populations. In-vitro experiments demonstrated a decreased response to *Aspergillus* antigens in monocytes homozygous for the Dectin-1 Y238X mutation. No in-vitro data was generated with regard to the response in macrophages heterozygous for the Dectin-1 Y238X mutation since the in vitro assays were only performed to find a mechanistic explanation of the limited influence of this Dectin-1 polymorphism on susceptibility to IA. Nonetheless, whether a diminished function after exposure to Aspergillus would also occur in a patiente heterozygote for the Y238X mutation would be interesting to study in the in-vitro setting. In general, previous research on the effects of mutations in genes encoding cell surface receptors showed that a decrease or increase in function can be also expected in the heterozygotes [15, 16].

Other polymorphisms in genes coding for components of the innate immunity to *Aspergillus* infections have been recently reported to increase susceptibility to disease caused by this pathogen: *TLR1*, *TLR4*, *TLR6, IL1* and the *IL10* promoter region [17-20]. In all of these studies, the polymorphism of interest was studied in isolation and not in association with each other. Thus, the relative influence of combinations of polymorphisms was not addressed. Simultaneous presence of two or more of these polymorphisms in a patient may further enhance the risk profile to IA. In the case-control investigation described in **chapter 7**, we found that the *TLR4* 1063A>G polymorphism was associated with increased susceptibility to IA, when present in the donors DNA of ASCT recipients (alone or in combination with *TLR6* 745C>T or *IFNG*

874T>A SNPs). The *IFNG* 874T>A SNP appeared to enhance the risk conferred by two of the TLR polymorphisms. Although carriers of this genetic variation produce suboptimal levels of IFN-γ, putting them at increased risk for perhaps manifest mycobacterial infection [16], the isolated presence in either donor or recipient did not increase the risk for IA. Remarkably, SNPs that affect the production of IL-10, one of the most important broad-acting negative modulators of the TLR to IL-12 and IFN-γ macrophage-activating pathway, were not associated with IA.

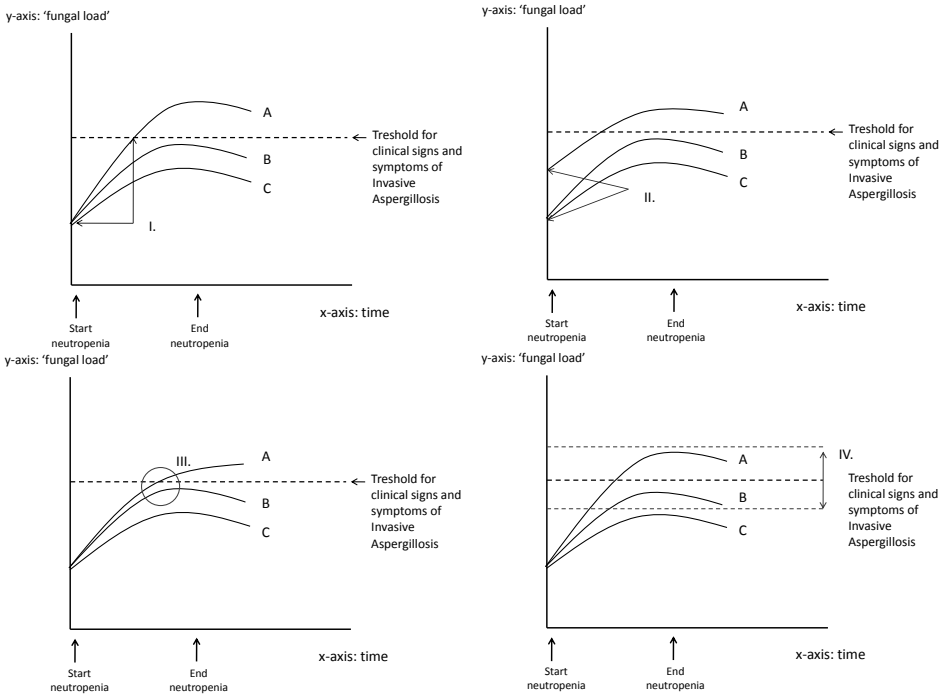
Compared to other risk factors, the absolute risk conferred by relevant SNPs in PRR- and cytokine genes seems limited. Healthy individuals carrying these SNPs do not develop IA unless a profound immune deficiency is present. It is more probable that specific patterns of genetic polymorphisms rather than a single genetic variation in TLRs or subsequent cytokine pathways that activate macrophages may be associated with IA in patients at risk. The observation of the association between the *TLR4* 1063A>G plus *IFNG* 874T>A SNP combination in our study fits this hypothesis.

On the other hand, probable associations of IA with conditional combinations of mutations may also attest to the complex immuno-pathogenesis of IA. As a consequence of neutropenia limiting the redundancy in the immune response to IA, the role of key components within the innate immune response could be more prominent in the remaining defense against invasive fungal infection and thus facilitate linkage to TLR- in combination with cytokine SNPs. Assuming that the studied SNPs have an effect on the functioning of the innate immune system, different SNPs may also be working at different time points to modulate resistance to IA and eventually constitute the overall genetic signature of susceptibility (figure 2).

The potential of (future) serum markers for the diagnosis of pulmonary fungal infection.

In the prospective study described in **chapter 5**, we demonstrated that serum 1,3-β-D-glucan (β-D-glucan) - but not serum S-adenosylmethionine (AdoMet) - was an accurate diagnostic tool for the diagnosis of PCP in HIV-negative immunocompromised adults. In HIV-infected individuals, the clinical relevance of serum β-D-glucan has already been investigated in larger studies [21]. These studies showed a sensitivity and specificity that was not surpassed by other potential markers, except may be by AdoMet [22, 23]. Several major concerns preclude transposing the results found in HIV-positive populations with PCP to the HIV-negative population at risk for PCP. First of all, autopsy studies reported lower loads of *P. jirovecii* in the lungs of immunocompromised patients without HIV as compared to HIV-positive patients with PCP [24, 25]. With a lower pulmonary fungal burden, the amount of cell wall components of the Pneumocysts that enter the circulation are probably smaller. In addition, patients at risk for PCP, but with a relatively more fit immune system may have a less slow decrease of circulating β-D-glucan [26]. In an observational study performed by Nakamura et al., serum β-D-glucan levels were confirmed to be significantly lower in patients with PCP due to other underlying

Figure 2. Hypothetical biological determinants of development of IA that are potentially influenced by host genetic polymorphisms.



Legend: Line A, B and C represent individual hosts susceptible to IA. Diagram I-IV shows the spectrum of mechanisms that can be involved. Genetic polymorphisms may affect the functioning of the remaining lung macrophages and epithelial cells at the start of neutropenia (I); influence the level of colonization of the airways and alveoli with Aspergillus prior to the neutropenic period (II); influence actions of lung epithelial cells and macrophages or neutrophils throughout the period at risk (III); or affect factors that regulate the development of symptoms (IV). Modified after: human variations in susceptibility to infection by *S.typhi*: evidence from the distribution of incubation periods in single-exposure epidemics. van Dissel J.T . & van Furth R. (1993); In: Cabello F, ed. *The Biology of Salmonella; Proceedings of NATO ASI*. (pp 385-389).

causes than HIV [27]. Secondly, HIV-negative patients at risk for PCP – and the population of patients with hematologic disorders in particular – generally have a higher a priori chance to develop other invasive pulmonary fungal infections, e.g. invasive aspergillosis or candidemia. Due to the presence of β-D-glucan in the cell wall of these organisms, a false positive test result may be obtained when only the 1 β-D-glucan test is used to diagnose PCP. Hence, the use of the β-D-glucan assay as a single test for the purpose of diagnosing PCP is clearly limited [28]. Thus, a primary suspicion of PCP above other fungal infections (supported by clinical signs and symptoms as well as chest imaging) seems warranted. Careful assessment of the clinical presentation and chest imaging, remain to play an important role in the diagnostic work up. Furthermore, we found that follow-up levels of β-D-glucan significantly decreased over relatively short time during treatment. However, the values measured after a median of 3 days of treatment still remained far above the upper limit of normal in >90% of cases, indicating that for now the clinical follow up is of is of at least equal importance when determining the response to treatment.

Despite of previous reports claiming AdoMet to be both a highly sensitive and a specific marker for PCP in HIV-positive patients, this could not be confirmed in our study in a HIV-negative population with PCP. Concerns similar to those expressed above on the reliability of the β-D-glucan test exist with regard to AdoMet. The suggested mechanism by which AdoMet would be useful as a marker for PCP is its depletion from the serum during infection with *P. jirovecii* [29]. Since lower pulmonary fungal loads are present in HIV-negative patients with PCP, this may adversely influence the serum AdoMet level in a way that its reliability as a diagnostic test for PCP becomes compromised. Moreover, AdoMet is the product of the human body's own metabolism and other factors, e.g., malnourishment, general clinical condition and other variables are known to affect the level of serum S-adenosylmethionine [30]. Our study is the first that assessed the reliability of this marker in HIV-negative patients. Unfortunately, we found that it failed to discriminate between HIV-negative immunocompromised patients with and without PCP.

As described in **chapter 8** specific tracers, i.e. radiolabeled antimicrobial peptides, fluconazole and agents targeting chitin, may prove useful for the diagnosis of invasive fungal infections in the near future. The main limitation of usage of radiolabeled antimicrobial peptides - that appear to discriminate between infections and sterile inflammatory processes - is their inability to distinguish fungal infections from bacterial infections. However, these markers may be suitable for other purposes. For example, radiolabeled antimicrobial peptides were successful in monitoring antifungal therapy in *C. albicans*-infected mice. One step further, radiolabeled fluconazole distinguished *C. albicans* infections from bacterial infections and sterile inflammatory processes, and failed to image *A. fumigatus* infections. The challenge now is to bring promising markers through the phase I to III clinical trials that ensure their safety as well as to assess their added value as a diagnostic clinical test.

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Chapter 10

NEDERLANDSE SAMENVATTING

Inleiding

Ten gevolge van de toepassing van orgaan- en stamceltransplantatie als behandeling voor ziekten waarvoor eerder geen therapeutische opties meer bestonden, neemt het aantal personen met een ernstig in kwaliteit verminderd afweersysteem wereldwijd toe. De aantasting van het afweersysteem wordt bij deze patiënten veroorzaakt door de medicatie die wordt toegediend ter voorkoming van afstoting van het getransplanteerde orgaan of door de behandeling met intensieve chemotherapie voorafgaand aan stamceltransplantatie. Hierdoor ontstaat gedurende langere tijd een verhoogde vatbaarheid voor infecties. In een dergelijke situatie kunnen complicerende infecties ook veroorzaakt worden door zogenaamde opportunistische pathogenen. Dit zijn micro-organismen die alleen in staat zijn om ziekte te veroorzaken als het immuunsysteem ernstig is verzwakt.

De in dit proefschrift beschreven studies gaan over het optreden van opportunistische schimmelinfecties van de longen na transplantatie. Deze infecties gaan gepaard met een hoge sterfte. In West-Europa zijn *Pneumocystis jirovecii* (box 1) en *Aspergillus* soorten (box 2) de meest voorkomende verwekkers van een dergelijke infectie. Welk type schimmelinfectie zich voordoet - en op welk tijdstip - wordt grotendeels bepaald door de aard van onderliggende aandoening en de voorafgaande behandeling van de patiënt. Deze twee factoren bepalen namelijk zowel de aard als de ernst van de ontstane gebreken in het afweersysteem. In geval van uitschakeling van de door T-lymfocyten gegenereerde afweerrespons is de patiënt vooral vatbaar voor het ontwikkelen van een pneumonie (longontsteking) veroorzaakt door *Pneumocystis jirovecii*. Bij langer durende afwezigheid van neutrofiele granulocyten (een subtype witte bloedcellen) treden vooral longinfecties op met *Aspergillus* als verwekker.

Box 1 *Pneumocystis*

Het micro-organisme *Pneumocystis* werd gedurende lange tijd na zijn beschrijving (in ratten) door Delanoe & Delanoe in 1912, geclassificeerd als een protozoa. Pas kort na de 2e wereldoorlog ontdekten artsen dat *Pneumocystis* in staat was tot het veroorzaken van een ernstige interstitiele longontsteking bij mensen met een verstoorde afweer (door bijvoorbeeld ernstige ondervoeding, of chemotherapie of orgaantransplantatie). Ten gevolge van de HIV-epidemie, en de daarmee samenhangende stijging van het aantal patienten met een *Pneumocystis pneumonie*, kwam het onderzoek in een stroomversnelling. Er bestaan meerdere soorten *Pneumocystis*, en elk afzonderlijke soort heeft zijn eigen gastheer. De soort die longontsteking bij de mens veroorzaakt was voorheen bekend als *Pneumocystis carinii* of *Pneumocystis carinii f. sp. hominis*, maar is recent omgedoopt tot *Pneumocystis jirovecii*. Het bleek ondermeer dat *Pneumocystis jirovecii* ook de longen kan 'koloniseren' van gezonde personen zonder evident ziekte te veroorzaken. Alleen in geval van het dysfunctioneren van de T-lymfocyt gerelateerde immunteit kan een ernstige interstitiele longontsteking ontstaan. Op basis van DNA-analyse en op grond van met elektronenmicroscopie nieuw ontdekte morfologische kenmerken van *Pneumocystis* worden *Pneumocystis* soorten momenteel geclassificeerd als schimmels.

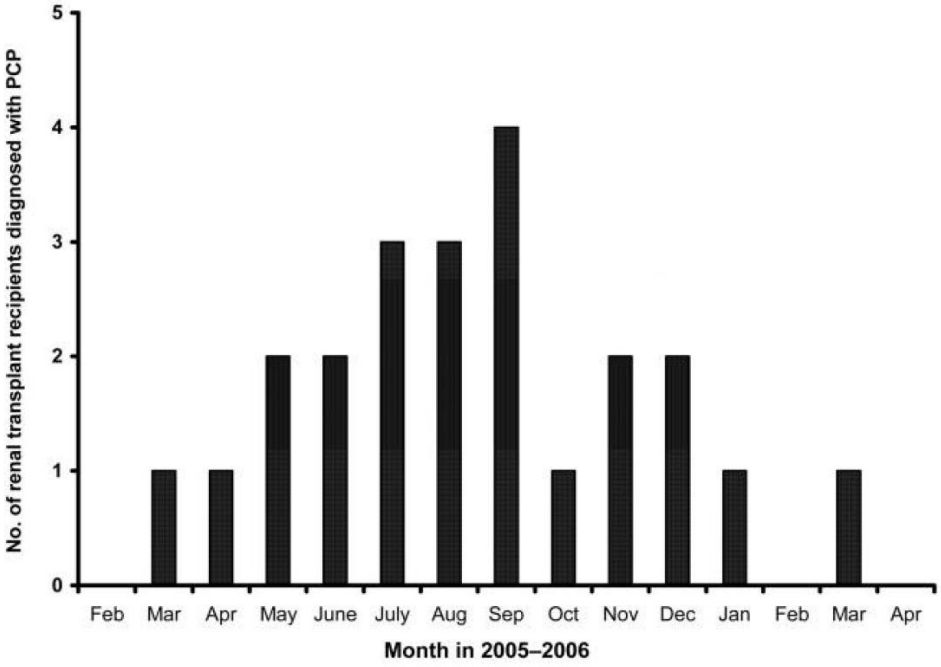
Box 2. *Aspergillus* en 'invasive aspergillose'
De term 'invasieve aspergillusinfectie' verwijst naar weefselinvasie door de filamenteuze schimmel *Aspergillus*. Er bestaan diverse ondersoorten, waarvan *A. fumigatus*, *A. flavus*, *A. niger* en *A. terreus* het meest frequent verantwoordelijk zijn voor ziekte bij de mens. *Aspergillus* is wereldwijd op grote schaal aanwezig in de bodem, in voedsel en in vochtige omgevingen. De sporen (conidia) worden verspreid via de lucht en kunnen na inademing direct aanleiding geven tot verschillende aandoeningen van de luchtwegen. Invasieve infecties van de longen komen echter alleen voor bij patienten met een ernstig gestoord functioneren (of bij afwezigheid-) van de witte bloedcellen, in het bijzonder de neutrofiele granulocyten. De longen of het KNO-gebied zijn de primaire locatie van de infectie (in 90-95% van alle gevallen). Via de bloedsomloop kan Aspergillus dissemineren naar andere organen, met name ook naar de hersenen.
De erkenning als één van de belangrijkste infectieuze complicaties na een stamceltransplantatie is een belangrijke drijvende kracht achter het klinische en experimentele onderzoek naar Aspergillus. Veel risicofactoren zijn inmiddels bekend en er zijn een aantal markers beschikbaar om een invasieve aspergillusinfectie vroegtijdig te diagnosticeren. Ondanks dit alles heeft de huidige incidentie van deze ziekte zich gestabiliseerd op ongeveer 5-8% van alle patiënten die behandeld worden door middel van allogene stamceltransplantatie.

Dit proefschrift

In **hoofdstuk 2** beschrijven we het onderzoek dat werd verricht naar een *Pneumocystis*-pneumonieuitbraak onder niertransplantatiepatiënten in het Leids Universitair Medisch Centrum (figuur 1). Hierin komen alle facetten van een klassiek epidemiologisch onderzoek naar een uitbraak van een infectieziekte aan bod. Er waren al langere tijd zowel directe aanwijzingen (dierexperimenteel onderzoek) als indirecte aanwijzingen dat dit micro-organisme van patiënt naar patiënt kan worden overgedragen. Nu deed zich de mogelijkheid voor om dit in de praktijk te onderzoeken met behulp van nieuwe pathogeen genotyperingsmethoden en aan de hand van nauwkeurige epidemiologische gegevens. De resultaten van de genotypering laten zien dat het overgrote deel van de patienten een *Pneumocystis* pneumonie doormaakte die werd veroorzaakt door hetzelfde *Pneumocystis* genotype (d.w.z.: met hetzelfde DNA profiel). Tevens bleken de patienten op dezelfde momenten de polikliniek nierziekten bezocht te hebben, of tegelijkertijd met een andere patiënt opgenomen te zijn geweest op de klinische afdeling. Dit is suggestief voor de overdracht van het pathogeen van patiënt naar patiënt.

Ter verdere exploratie werden de beschikbare epidemiologische gegevens met statistische modellen geanalyseerd. Hiermee kon overdracht van *P. jirovecii* van patiënt tot patiënt wel aannemelijk gemaakt worden, maar een externe bron kon niet worden uitgesloten. Nadeel van dergelijke modellen blijft dat zij slechts een zeer abstracte weergave zijn van de werkelijkheid.

Figuur 1. Grafische weergave van het aantal nieuwe niertransplantatiepatienten met een *Pneumocystis* pneumonie per maand.



Verder werd een systematisch literatuuronderzoek verricht naar het ontstaan van *Pneumocystis* pneumonie uitbraken onder patienten met een niertransplantaat (**hoofdstuk 3**). Er werden 15 wetenschappelijke artikelen geïncludeerd. Uit de analyse bleek ondermeer dat in de medische centra waar de epidemieen vanaf 1980 plaatsvonden geen chemoprofylaxe ter voorkoming van *Pneumocystis* pneumonie werd voorgeschreven. Verder was er bijna altijd sprake van contacten tussen de patienten in de periode voorafgaande aan de diagnose. Bij opname werden de patienten met een *Pneumocystis* pneumonie niet geïsoleerd. De gerapporteerde klinische risicofactoren verschilden niet van de risicofactoren die gevonden werden in twee case-controle onderzoeken waarbij geen sprake was van een epidemie. Per 10 jaar daalde de gerapporteerde mortaliteit aanzienlijk van gemiddeld 38% voor 1990 naar 19% tussen 1990 en 2000, en vervolgens naar een gemiddelde van 13% tussen 2000 en 2010. Dit heeft meerdere oorzaken. Ten eerste heeft sinds circa 1990 het toevoegen van steroïden aan het behandelingsschema de overleving van patienten met een *Pneumocystis* pneumonie sterk verbeterd. Daarnaast zijn een betere bekendheid van het ziektebeeld en een door de tijd heen verbeterende intensive care zorg waarschijnlijk belangrijke bijdragende factoren. Het moment van ontstaan van de epidemieen was niet evident gerelateerd aan een bepaald jaargetijde. De combinatie van de epidemiologische gegevens en de resultaten van de genotypering van de aangetroffen *Pneumocystis* pleitte in bijna alle studies voor de overdracht van *P. jirovecii* van patiënt naar patiënt. Hoewel dergelijke uitbraken zeldzaam zijn, dient

men om deze (maar ook individuele gevallen) te voorkomen, antibiotische profylaxe voor te schrijven. De optimale duur van deze antibioticaprofylaxe gerekend vanaf het moment van transplantatie is onbekend. De huidige van toepassing zijnde richtlijnen geven hierover uiteenlopende adviezen.

In **hoofdstuk 4** wordt getracht een antwoord te vinden op de vraag hoe lang een individuele patiënt na niertransplantatie ter voorkoming van een *Pneumocystis* pneumonie antibioticaprofylaxe zou moeten innemen. Er kleven ook nadelen aan het innemen hiervan, zoals bijwerkingen, beïnvloeding van de nierfunctie, maar ook het in de hand werken van antibioticaresistentie bij verscheidene micro-organismen is een punt van zorg. Dit onderzoek voltrok zich in twee fasen. Als eerste werd in een case-controle onderzoek onderzocht welke klinische factoren de kans op een *Pneumocystis* pneumonie vergroten. De onderzochte groep patiënten bestond uit 50 niertransplantatiepatiënten met *Pneumocystis* pneumonie en 99 niertransplantatiepatiënten zonder *Pneumocystis* pneumonie. In een multivariate analyse werd vervolgens ook de samenhang van de gevonden risicofactoren bestudeerd. Het bleek dat op het moment van transplantatie een oudere leeftijd (55+) een iets meer dan 2,5-maal extra kans gaf op het ontwikkelen van een *Pneumocystis* pneumonie. Verder waren ook behandeling voor afstoting van het niertransplantaat (met hoge dosis prednison of antithymocyten globuline) en CMV infectie geassocieerd met het ontwikkelen van een *Pneumocystis* pneumonie (respectievelijk c.a. 6x en 3x zo vaak in vergelijking met de controlegroep). De wijze waarop het immuunsuppressieve medicatieprofiel was samengesteld bleek niet van invloed. Dit is in tegenstelling tot wat eerder in de literatuur op basis van case-series en op grond van onderzoek bij ratten werd gesuggereerd. Een belangrijke bevinding was verder dat 85% van de patienten met een *Pneumocystis* pneumonie zich presenteerde tussen de 2^{e} en 24^{e} maand na niertransplantatie. Bij nadere studie bleek dat in de overige 15% van de patienten er vrijwel altijd een bijzondere omstandigheid aanwezig was waardoor de vatbaarheid voor het krijgen van *Pneumocystis* pneumonie ook na de 24^{e} maand verhoogd was. De verkregen gegevens werden vervolgens in het 2^{e} deel van de studie gebruikt in een rekenmodel waarmee de effectiviteit van een aantal profylaxestrategieën (gebaseerd op de geïdentificeerde risicofactoren) werd geschat. Hieruit bleek dat voor patienten zonder risicofactoren een behandelduur tot 6 maanden na transplantatie voldoende is. Indien sprake is van een leeftijd ouder dan 55 jaar ten tijde van transplantatie, of indien rejectiebehandeling moet worden toegepast, is verlenging van de duur van de profylaxe tot 1 jaar na transplantatie aan te bevelen. Het is van belang te realiseren dat deze schattingen gebaseerd zijn op retrospectief verkregen gegevens. De effectiviteit van deze strategieën moet daarom ook verder prospectief worden onderzocht.

Het vroegtijdig stellen van de diagnose *Pneumocystis* pneumonie is van groot belang. Voor het met zekerheid stellen van de diagnose moet - volgens de huidige standaarden - materiaal uit de diepere luchtwegen worden verkregen. Dit kan door middel van een broncho-alve-

olaire lavage of een longbiopsie. Dit materiaal wordt dan onderzocht op de aanwezigheid van *P. jirovecii* door middel van directe microscopie en toepassing van PCR-technieken. Beide onderzoeksprocedures ter verkrijging van het materiaal zijn belastend voor de patiënt en geven een niet te verwaarlozen kans op complicaties. In **hoofdstuk 5** beschrijven we het onderzoek dat werd verricht naar het toepassen van een patiëntvriendelijkere methode om de diagnose *Pneumocystis* pneumonie te stellen: namelijk door het meten van zogenaamde 'markers' in het bloed. Twee chemische verbindingen kwamen hiervoor in aanmerking: S-adenosylmethionine (AdoMet) en 1,3-β -D-glucan (β-D-glucan).

Het S-adenosylmethionine is een lichaamseigen stof die door de cellen van het lichaam wordt gebruikt bij diverse metabole processen. Onderzoek bij ratten laat zien dat tijdens een infectie met *P. jirovecii* de concentratie van deze stof in het serum fors daalt. De hypothese bestaat dat *P. jirovecii* niet in staat is tot het zelf aanmaken van AdoMet en afhankelijk is van opname uit de omgeving. Enkele uitzonderingen daargelaten zijn verder alle parasieten, schimmels en bacterien wel in staat tot de synthese van AdoMet. In tegenstelling tot AdoMet is het β-D-glucan niet een lichaamseigen stof, maar een belangrijk onderdeel van de celwand van *P. jirovecii*. Bij het uit elkaar vallen van het micro-organisme komt het β-D-glucan vrij in de bloedcirculatie. Een detecteerbare concentratie van β-D-glucan in het serum zou derhalve wijzen op de diagnose *Pneumocystis* pneumonie.

De door ons uitgevoerde studie was gericht op de evaluatie van de hierboven beschreven markers bij HIV-negatieve patienten met een mogelijke *Pneumocystis* pneumonie. Uit autopsiestudies is gebleken dat in deze groep patienten de hoeveelheid *Pneumocystis* relatief lager is - en de inflammatie in de longen heviger - in vergelijking met Hiv-geïnfecteerde patiënten met een *Pneumocystis* pneumonie. Eerdere studies beoordeelden de diagnostische waarde van de genoemde serum markers slechts bij Hiv-positieve patiënten. Deze resultaten zijn dus niet zondermeer toepasbaar op patienten met bijvoorbeeld een solide orgaan transplantaat en een verdenking op *Pneumocystis* pneumonie. Uit onze studie bleek dat de serum AdoMet waarden niet goed discrimineerden tussen het hebben van wel of geen *Pneumocystis* pneumonie op het moment van presentatie in het ziekenhuis. Daarentegen was het onderscheidende vermogen van de β-D-glucan serum marker wel zeer goed. De hoogte van de concentratie gemeten β-D-glucan hing tevens significant samen met de surrogaatwaarde voor de hoeveelheid *Pneumocystis* DNA in de long, de zogenaamde Ct-waarde. Nadeel van de β-D-glucan test blijft dat deze ook in het geval van sommige andere schimmelinfecties een positieve uitslag geeft. Dit maakt dat deze test alleen als diagnosticum voor *Pneumocystis* pneumonie gebruikt kan worden als hierop een hoge klinische verdenking bestaat op basis van zowel het onderliggende lijden en de radiologische beeldvorming (bij voorkeur CT-scan).

Het 2e deel van dit proefschrift bevat de beschrijving van twee onderzoeken naar de genetische predispositie voor het ontwikkelen van een invasieve aspergillusinfectie bij patienten die een allogene stamceltransplantatie hebben ondergaan. Bij afwezigheid of slecht functioneren

van de witte bloedcellen na de voorbereidende chemotherapie voor stamceltransplantatie is de patiënt kwetsbaar voor invasieve schimmelinfecties. Een afweerbarrière die dan nog wel kan worden opgeworpen is die van de zogenaamde 'innate immunity' (aangeboren afweer). Onderdeel hiervan zijn de in de long aanwezige macrofagen (een bepaald type afweercellen) die met behulp van receptoren binnenkomende schimmelkiemen (conidia) herkennen en afbreken. Ook kunnen zij door uitscheiding van signaalstoffen (cytokines) andere afweercellen uit de omgeving aantrekken om de ontstekingreactie te intensiveren.

In het onderzoek beschreven in **hoofdstuk 6** onderzochten we of een defecte Dectin-1-receptor functie, ten gevolge van de stopcodon mutatie 'Y238X', het risico op het ontwikkelen van invasieve aspergillusinfectie verhoogd. De dectin-1 receptor is een eiwit dat dient voor het herkennen van β-D-glucan in de wand van een schimmel. Afweercellen van patienten die de Y238X-mutatie van zowel vaders als moederszijde hebben geërfd bezitten deze receptor niet. Het klinische belang van de aanwezigheid van deze mutatie werd geëvalueerd in een groep van 71 patiënten die een allogene stamceltransplantatie hadden ondergaan, en in een aparte groep van 21 patiënten die een invasieve aspergillus-infectie ontwikkelden na chemotherapie zonder stamceltransplantatie. De controlegroep bestond uit 108 patiënten die allen een allogene stamceltransplantatie hadden ondergaan. Tevens werd bestudeerd of de aanwezigheid van de Y238X-mutatie was gerelateerd met het beloop van de aspergillus-infectie. Bij patienten die een allogene stamceltransplantatie hadden ondergaan en heterozygoot waren voor de Y238X-mutatie bleek een trend aanwezig tot een hoger risico voor het ontwikkelen van een invasieve aspergillus-infectie. Het genotype van de stamceldonor was niet van invloed. Een opvallende bevinding was dat Y238X allelfrequentie hoger was bij niet-getransplanteerde hematologiepatiënten met een invasieve aspergillus-infectie (19.0%) ten opzichte van het wél getransplanteerde patiëntencohort en een steekproef uit de Nederlandse bevolking (bereik 6.9%-7.7%, pH-waarde <0.05). Uit laboratoriumtesten bleek dat uit het bloed gewonnen cellen met een volledig defect in Dectin-1 functie minder efficiënt reageerden tegen *Aspergillus*. Echter, gekweekte macrofagen met dezelfde deficiëntie, bleken ondanks de gebrekkige Dectin-1 functie wel een adequate reactie te kunnen genereren tegen *Aspergillus*. Dit laat zien dat het aangeboren afweersysteem vermoedelijk beschikt over alternatieve oplossingen in geval van het disfunctioneren van één of enkele afzonderlijke componenten.

Hoofdstuk 7 beschrijft het onderzoek naar de invloed van de gemeenschappelijke aanwezigheid van mutaties in Toll-like receptor- en cytokine genen op het risico voor het ontwikkelen van een invasieve aspergillus-infectie. De genoemde genen zijn allen betrokken bij de regulatie van de activiteit van longmacrofagen. Vierenveertig patienten die een allogene stamceltransplantatie ondergingen en bij wie een invasieve aspergillus-infectie werd gediagnosticeerd, werden in het onderzoek geïncludeerd. De controlegroep bestond uit 64 allogeen getransplanteerde patienten zonder invasieve aspergillus-infectie. Alle patienten hadden een onderliggende hematologische ziekte. De aanwezigheid van de 1063 A>G

mutatie in Toll-like receptor 4 bleek alleen geassocieerd met het ontstaan van een invasieve aspergillus-infectie indien deze mutatie aanwezig was bij de donor van de getransplanteerde patiënt. In een multivariate analyse was de gecombineerde aanwezigheid van de Toll-like receptor 4 1063 A>G en de Interferon-γ 874 T>A *of* de gecombineerde aanwezigheid van de Toll-like receptor 6 745 C>T met de Interferon-γ 874 T>A mutatie, geassocieerd met het optreden van invasieve aspergillus-infectie. Hoewel de resultaten ook na correctie voor aanwezigheid van graft-versus-host ziekte en de duur van de neutropenie significant waren of een duidelijke trend lieten zien, is er een aantal beperkingen aan deze studie. Vooral door de relatief kleine aantallen patienten kunnen de resultaten vertekend zijn. De bevindingen wijzen op de relevante immunologische routes die betrokken zijn bij afweer tegen invasieve aspergillus-infectie van de longen en rechtvaardigen verdere studie naar de effecten van Toll-like receptor en cytokine mutaties.

Hoofdstuk 8 geeft een overzichtsbeschrijving van de huidige stand van zaken ten aanzien van de ontwikkeling van radioactiefgelabelde antimicrobiële moleculen die mogelijk gebruikt kunnen worden voor het diagnosticeren van invasieve schimmelinfecties. Dit onderzoek is grotendeels nog in een experimentele fase. De belangrijkste studies in muizen laten vooral zien dat Technetium-99 gelabeld fluconazol een selectieve tracer kan zijn voor het detecteren van *Candida albicans* infecties. Voor *Aspergillus* zijn dergelijke proeven tot op heden niet erg succesvol gebleken. In de toekomst zouden nieuwe antimicrobiele medicamenten, bijvoorbeeld de echinocandines, voor dit doel geëvalueerd kunnen worden. Uiteindelijk zullen de in klinische studies onderzochte reproduceerbaarheid en veiligheid, van deze nieuw te ontwikkelen markers bepalen of zij kunnen worden toegevoegd aan het diagnostisch armamentarium.

Conclusies

Het onderzoek uit het eerste deel van het proefschrift toont aan dat de overdracht van *Pneumocystis jirovecii* meest waarschijnlijk plaats vindt van persoon tot persoon. In de engelse samenvatting en discussie worden de achterliggende overwegingen ten aanzien van de factoren die een rol spelen in het ontstaan van een uitbraak/epidemie uitgebreid uiteengezet. Verder benadrukken de bevindingen het belang van het toepassen van een profylaxestrategie ter voorkoming van een *Pneumocystis* pneumonie bij niertransplantatiepatienten. De resultaten ondersteunen ook het eerder veronderstelde nut van isolatiemaatregelen bij patienten die worden opgenomen met een *Pneumocystis* pneumonie.

Ten aanzien van de duur van de profylaxe zijn er meerdere benaderingen mogelijk. Om de incidentie van *Pneumocystis* pneumonie onder niertransplantatiepatienten laag te houden (beneden de 1 per 100 transplantaties) kan gekozen worden voor een duur tot 12 maanden na transplantatie voor iedere patiënt, of voor een risicoprofiel geleid algoritme. In het meest

optimale algoritme krijgen alleen de relatief oudere patienten (55+) en diegenen die behandeld worden voor afstoting van het transplantaat tot 12 maanden na transplantatie cotrimoxazol. Alle overige patienten behoeven slechts profylaxe te gebruiken tot 6 maanden na transplantatie.

Het onderzoek naar het gebruik van serummarkers voor de diagnose *Pneumocystis* pneumonie bij niet Hiv-geinfecteerden laat zien dat β-D-glucan het meest geschikt is voor dit doel. Een juiste beoordeling van de à a-priori kans op basis van de klinische en radiologische gegevens is van groot belang om de uitslag juist te kunnen interpreteren. Het gebruik van radioactief gelabelde stoffen voor het diagnosticeren van invasieve schimmelinfecties is nog in het stadium van dierexperimenteel onderzoek. Een aantal vorderingen zijn veelbelovend, maar behoeven in de toekomst verdere evaluatie in klinische trials.

Uit de studies naar de genetische predispositie voor het ontwikkelen van een invasieve aspergillus-infectie na stamceltransplantatie blijkt dat geen enkele mutatie in het innate immuunsysteem van essentieel klinisch belang is. Aanwezigheid van de Dectin-1 Y238X mutatie heeft bijvoorbeeld slechts een beperkte invloed op de kans op het ontstaan van een invasieve aspergillus-infectie. Combinatie van verscheidene mutaties zoals hierboven beschreven lijken, met name indien aanwezig in het DNA van de stamceldonor, wel degelijk relevant. Meer uitgebreide, prospectieve onderzoeken zijn nodig om deze bevindingen te bevestigen.

Curriculum Vitae

Mark de Boer werd geboren op 25 februari 1977 te Utrecht. Na het behalen van het VWO diploma in 1995 begon hij met de studie geneeskunde in Leiden. Gedurende de studie was hij werkzaam als student-assistent bij de vakgroepen Celbiologie en Anatomie, en deed onderzoek op de neonatologie-IC van het Leids Universitair Medisch Centrum onder leiding van Prof. dr. F.J. Walther en dr. A.J. de Beaufort. Later volgden een 3 maanden durende klinische stage en een wetenschappelijk onderzoek in een ruraal gebied in Malawi onder supervisie van drs. J. de Witte (kinderarts), drs. E. Noorlander (tropen- en huisarts) en dr. A.M. Polderman (medisch parasitoloog). Na de co-schappen werd tevens een onderzoek uitgevoerd naar het immuunreconstitutiesyndroom bij HIV-geinfecteerde personen, begeleid door dr. F.P Kroon, zijn latere co-promotor. Gedurende de bovengenoemde ervaringen, als ook tijdens het co-schap Interne Geneeskunde in het Groene Hart ziekenhuis te Gouda, werd het de auteur geleidelijk duidelijk dat Interne Geneeskunde de specialisatie van keuze was. Na het behalen van het artsexamen (*cum laude*) aan de Rijks Universiteit Leiden in 2001, begon hij als arts-assistent op de afdeling Interne Geneeskunde van het Leids Universitair Medisch Centrum. Na aanname voor de opleiding tot internist (opleiders Prof. dr. A.E. Meinders, dr. J.W. van 't Wout en Prof. dr. J.A. Romijn) volgde in 2008 de registratie in het specialistenregister. Gedurende de periode van 2004 tot 2007 was hij bestuurslid van de Jonge Nederlandse Internistenvereniging (JNIV) en lid van het Forum Visitatorum. De vanaf het begin af aan gekoesterde interesse voor infectieziekten leidde in 2006 tot de keuze voor het betreffende aandachtsgebied (opleider Prof. dr. J.T. van Dissel), waarvoor hij registreerde in september 2008. Het onderzoek dat aanleiding gaf tot de totstandkoming van dit proefschrift nam in 2005 een aanvang, toen een uitbraak van *Pneumocystis* pneumonie onder niertransplantatiepatienten (beschreven in hoofdstuk 2) samenviel met een stage op de afdeling klinische epidemiologie onder leiding van Prof. dr. J.P. Vandenbroucke. Voor veel verkregen vaardigheden die nodig zijn voor het verrichten van klinisch onderzoek werd in deze periode de grondslag gelegd. Het onderzoek liep naast de klinische werkzaamheden van de auteur voort tot eind 2010. Behalve de speciale interesse in infecties veroorzaakt door *Pneumocystis jirovecii* en *Aspergillus sp.*, heeft de auteur zich verder toegelegd op de behandeling van patienten die geïnfecteerd zijn met HIV. Mark is getrouwd met Evelyne en heeft 2 lieve dochters, Rozemarijne en Pheline.

Nawoord

Dit proefschrift zou zonder de medewerking en inspanningen van velen niet zijn huidige vorm hebben bereikt.

Frank, ik weet nog goed de eerste keer dat ik op je werkkamerdeur klopte, 'of ik wat onderzoek mocht doen…?' Bedankt voor alles wat je me sindsdien leerde. Luc, ook mede dankzij jouw opgewekte aanmoediging en introductie in de 'Pneumocystologie' is het zover gekomen. Daarnaast dank ik de vele andere artsen, wetenschappers en overige medewerkers voor de fijne en vriendschappelijke samenwerking in de afgelopen jaren. Ook veel dank uiteraard aan alle patienten die deelnamen aan het onderzoek.

Een bijzonder woord van dank wil ik richten aan mijn ouders, die mij in staat stelden te studeren en het beroep te kiezen dat ik ambieerde.

Lieve Evelyne, Rozemarijne en Pheline, jullie zijn het geluk in mijn leven. Bedankt voor alle tijd, inspiratie en kracht die jullie mij gaven om dit project succesvol af te ronden. Laten we feestvieren en nog lang en gelukkig heel veel leuke dingen samen beleven.

List of Publications

de Boer MGJ, de Fijter JW, Kroon FP. Outbreaks and Clustering of Pneumocystis Pneumonia in Kidney Transplant Recipients: A Systematic Review. *Submitted*

de Boer MGJ, Jolink H, Halkes CJM, van der Heiden PLJ, Kremer D, Falkenburg JHF, van de Vosse, van Dissel JT. Influence of polymorphisms in innate immunity genes on susceptibility to invasive aspergillosis after stem cell transplantation. *Submitted*

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