Risk factors and new markers of pulmonary fungal infection

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Chapter 9

GENERAL DISCUSSION AND SUMMARY

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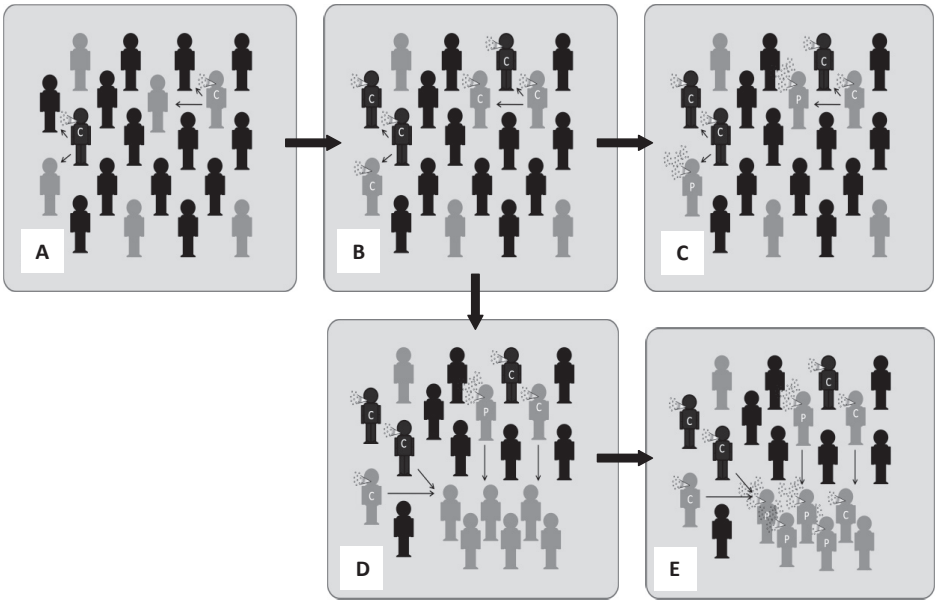
Pneumocystis pneumonia (PCP) and invasive aspergillosis (IA) are the most prevalent opportunistic pulmonary fungal infections occurring post transplantation. About both pathogens, but in particular about the in-vitro unculturable *P. jirovecii*, a high level of uncertainty exists with respect to transmission 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.

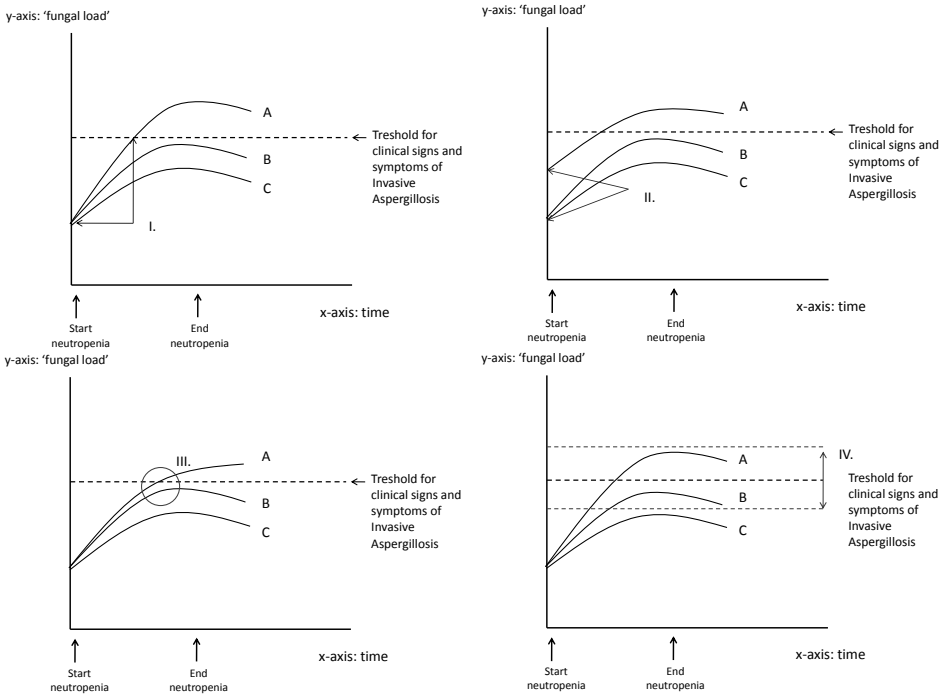
Compared to other risk factors, the absolute risk conferred by relevant SNPs in PRR- and cytokine genes seems limited. Healthy individuals carrying these SNPs do not develop IA unless a profound immune deficiency is present. It is more probable that specific patterns of genetic polymorphisms rather than 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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