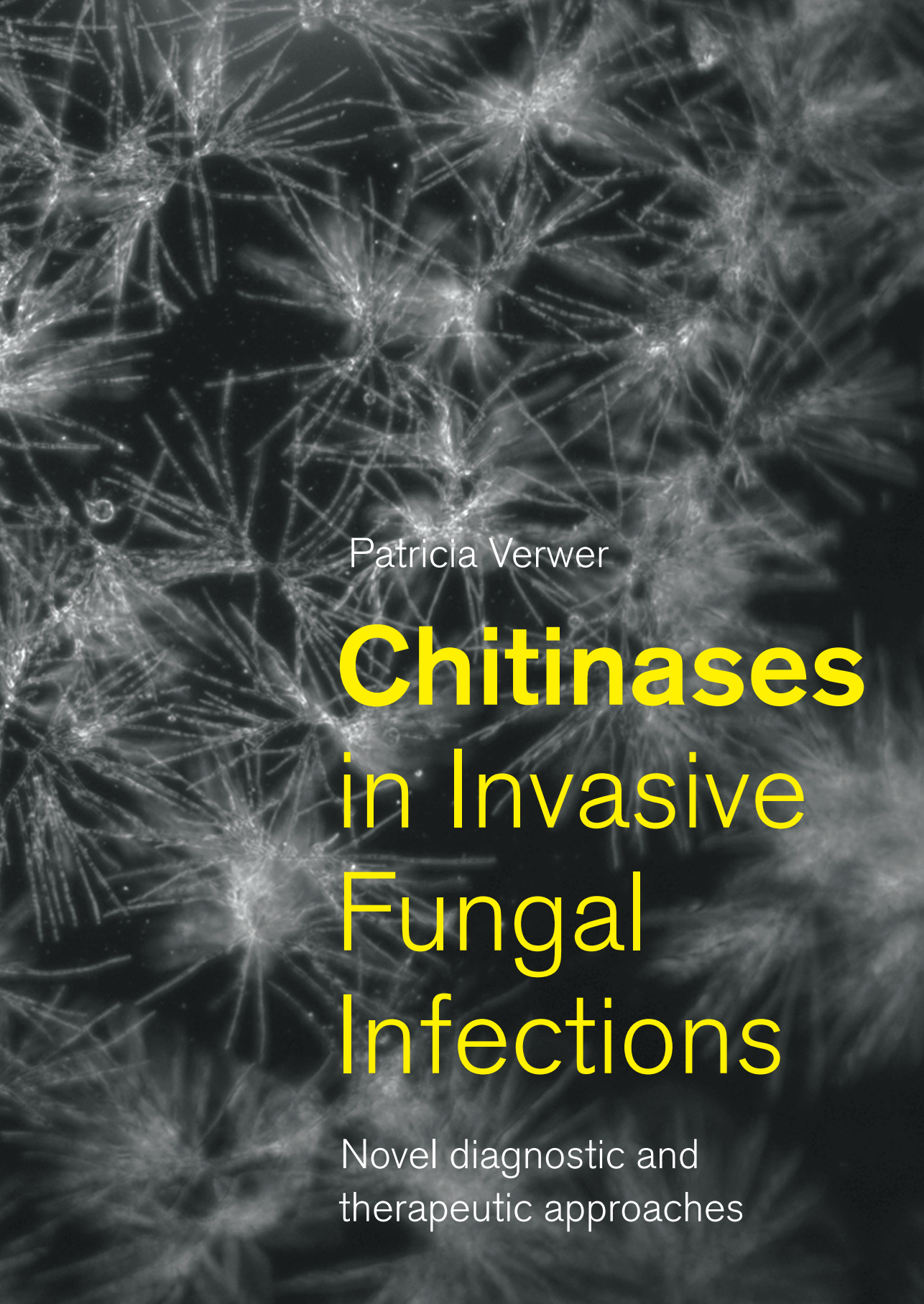

Patricia Verwer

Chitinases in Invasive Fungal Infections

Novel diagnostic and therapeutic approaches

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The printing of this thesis was financially supported by bioMérieux, ITK Diagnostics BV and VieCon.

Layout and printing: Optima Grafische Communicatie

Chitinases in Invasive Fungal Infections
Novel diagnostic and therapeutic approaches

Chitinases bij invasieve schimmelinfecties
Nieuwe diagnostische en therapeutische benaderingen

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 8 juni 2016 om 9.30 uur

Patricia Eline Bernadette Verwer
geboren te 's Gravenhage

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotor	Prof.dr. H.A. Verbrugh
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CHAPTER 1

Introduction and outline of the thesis

THE GENUS *ASPERGILLUS*, CLINICAL SYNDROMES AND CAUSATIVE AGENTS OF INVASIVE PULMONARY ASPERGILLOSIS

Aspergillus species (Figure 1 and 2) are ubiquitous fungi that are found all over the world. *Aspergillus* species are important in many commercial biological processes such as the production of citric acid, glucose oxidase and catalase (1, 2). Fungal spores of *Aspergillus* species are present in the open air and inside buildings. Concentrations inside buildings are relatively low, but can reach 10^4-10^7 per cubic meter in areas where compost is shredded (3). Humans and animals inhale fungal spores every day. Usually this remains without consequences when the immune system functions properly and the cells of the innate immune system among which macrophages attack and phagocytize the fungal spores. Epithelial cells and alveolar macrophages form the first barrier. Polymorphonuclear neutrophils are the second line of host defense to clear the fungal spores

Figure 1. Macroscopy of *A. fumigatus*. Photo by Loes van Damme-Rietvelt.

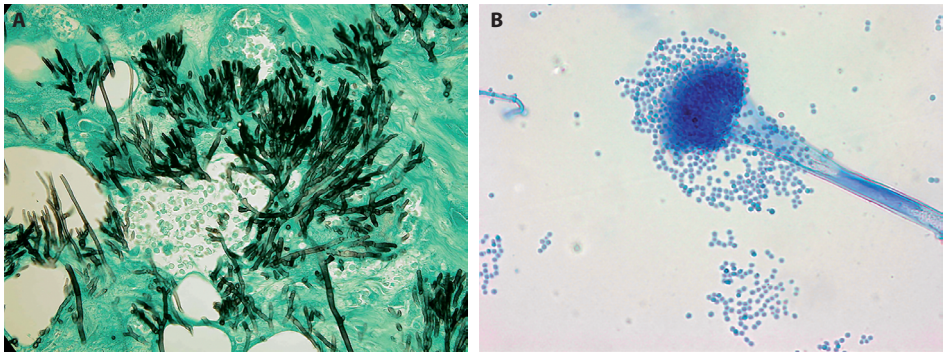

Figure 2. Microscopy of *A. fumigatus*. Photos by Loes van Damme-Rietvelt. A *A. fumigatus* in tissue, magnification ×40. Grocott staining. B *A. fumigatus in vitro* with conidiophore, magnification ×40. Lactophenol blue staining.

from the lungs (4, 5). As a result of this well-functioning innate immune response after inhalation of fungal spores, no disease develops (6).

In case of an overactive immune system, allergic airway disease can develop: allergic bronchopulmonary aspergillosis (ABPA)(7). This syndrome is characterized by immune-mediated airway damage, triggered by *Aspergillus* species after sensitization. ABPA is mainly found in asthma patients and in patients with cystic fibrosis. Patients with ABPA experience a worsening of their underlying asthma or a decrease in lung function (8).

On the other side of the spectrum of clinical disease, *Aspergillus* species may also cause invasive fungal infections. In these cases the immune response is not adequate or even absent and individuals are at risk for opportunistic invasive fungal infections (IFIs), including invasive pulmonary aspergillosis (IPA) (9). IPA is found exclusively in immunocompromised patients.

Over the past decades, the incidence of fungal infections has risen. In 2010 an incidence of over 178 cases per million population per year was reported (10, 11), however this might be an underestimation. From the several hundreds of *Aspergillus* species known, relatively few cause disease in humans. *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus lentulus* are among the pathogenic species, though *Aspergillus fumigatus* is found most frequently as causative agent of IPA (7).

PATHOGENESIS OF INVASIVE PULMONARY ASPERGILLOSIS

After inhalation of fungal conidia by a healthy person, several lines of defense protect the individual from becoming ill. Due to their small size of 2-3 µm, conidia reach the alveoli, where alveolar macrophages phagocytize the conidia. Conidia that have escaped the macrophages are attacked and destroyed by neutrophils and monocytes through the release of oxidants and degranulation (12). In an immunocompromised patient however, lines of defense are missing and disease develops. Conidia germinate into hyphae and an invasive type of fungal growth develops that disregards natural tissue barriers including the vasculature. Studies in neutropenic animal models show that a neutrophilic infiltrate is formed, followed by inflammation and a mononuclear cell infiltrate, ultimately leading to disseminated infection with hemorrhagic necrosis (7, 13).

DIAGNOSING INVASIVE PULMONARY ASPERGILLOSIS

Diagnosing fungal infections is usually not easy especially at the early stages of the infection. The first step in diagnosing an IFI is recognition of the patients at risk for the

infection, which is a heterogeneous group of patients. Not only patients with prolonged neutropenia are at risk, but also patients with advanced HIV-infection, with transplantations or with inherited immune deficiencies, as well as other critically-ill patients at intensive care units are prone to develop IFIs (9, 14, 15). The symptoms of fungal infections are non-specific signs of airway infection, like fever, dyspnea and coughing, which are not displayed by all patients due to impairment of the immune system (7, 15-18). When a patient is suspected of having an IFI, diagnostics are used to confirm the diagnosis. Patient material from normally sterile sites (blood, sputum, broncho-alveolar lavage fluid or lung biopsy) is sent for histopathological and microbiological evaluation in order to find the causative agent of the infection. Traditional diagnostic methods include microbiological techniques such as culture-based direct light microscopy, galactomannan assay (detecting the *Aspergillus* antigen galactomannan) and histopathology of biopsies (19). Traditional methods have several limitations. The sensitivity of cultures is relatively low (9, 15, 20) and a positive culture of e.g. sputum does not discriminate between colonization and infection. Also, identification to the species level can be difficult. Furthermore, diagnostic tests might be falsely negative in patients already receiving antifungal therapy. In some patients, at bronchoscopy the infected tissue in the lung might not be reached, resulting in falsely negative direct smears or needle biopsy. In other words, negative cultures do not exclude the possibility of invasive pulmonary aspergillosis (9, 15).

In the last years, molecular techniques have increasingly become available. These newer mostly PCR-based techniques offer several advantages compared to traditional methods, such as the ease of use, simplicity, short turnaround time and correct species identification (19, 20).

In addition to microbiological methods, imaging methods can also be helpful in the diagnostic route towards diagnosing an IFI. Radiologic investigations including chest X-ray and high resolution computed tomography (HR-CT) could show characteristic findings for invasive aspergillosis, such as the halo sign or air crescent sign (17, 21, 22). Based on clinical findings, histopathological and microbiological evaluation and radiological findings, patients can be classified as either proven, probable or possible cases of IPA according to the criteria defined by the European Organization for Research and the Treatment of Cancer-Mycoses Study Group (EORTC-MSG)(23). In brief, the criteria of "proven aspergillosis" are met when fungal material is cultured or observed microscopically in normally sterile material from direct biopsy or needle aspiration, in a patient with clinical signs of infection. A patient is classified as "probable aspergillosis" when a host factor (e.g. prolonged neutropenia), a radiological sign of fungal disease (e.g. halo sign, air-crescent sign) and a mycological factor (e.g. positive galactomannan index, positive culture) are present. Patients lacking a mycological factor are classified as "possible" aspergillosis, according to the definitions (23). This classification allows clinicians

to assess the risk of a patient to have aspergillosis. Early diagnosis of fungal infections is important, since treatment outcomes improve and healthcare costs are reduced when early and adequate therapy is started (9, 14, 24).

TREATMENT OF INVASIVE PULMONARY ASPERGILLOSIS

When a patient with IPA is diagnosed, the next challenge is treatment of the infection. Currently, IPA is mostly treated with voriconazole, based on data obtained in a randomized controlled trial that included 277 individuals with proven or probable aspergillosis (25). After publication of this study in 2002, no other randomized clinical trials with this high a number of patients have been published. Before this study was published, amphotericin B was the drug of choice for decades. Amphotericin B is a relatively old agent, belonging to the polyenes. It has a small therapeutic range due to a well-known dose-limiting nephrotoxicity (9). It binds to ergosterol in the fungal cell membrane and by forming pores, the permeability of the cell wall is increased, resulting in loss of essential nutrients and finally cell death. Amphotericin B was the first choice antifungal agent for a long time, but newer agents with lower toxic side effects have moved this agent downwards in the order of choice. The azoles, to which itraconazole, voriconazole and posaconazole belong, target 14-α demethylase and block the formation of ergosterol from its precursor lanosterol. Without ergosterol the fungal cell membrane and cell wall are not stable, resulting in cracks in the cell wall, ultimately leading to cell death. Azoles show less toxicity and voriconazole has become first choice in the treatment of aspergillosis (9, 14). The newest group of antifungal agents is the group of the echinocandins, to which caspofungin belongs, currently in use as salvage therapy.

CASPOFUNGIN, A DIFFERENT ANTIFUNGAL AGENT

Echinocandins have a different target than the polyenes and the azoles and act by the inhibition of synthesis of β-glucan, a major component of the fungal cell wall (Figure 3A and B)(26). In general, echinocandins are well tolerated and only few side effects were reported so far. Caspofungin is one of the echinocandins that is approved by the Food and Drug Administration for use in humans with IPA, though only as salvage therapy when patients are refractory or intolerant to other therapies (9, 14, 26). However, studies show that treatment with caspofungin in IPA is effective in terms of reduced mortality and cost effectiveness (27, 28).

The antifungal activity of caspofungin is different compared to that of other agents. When tested *in vitro*, low concentrations of caspofungin cause morphological altera-

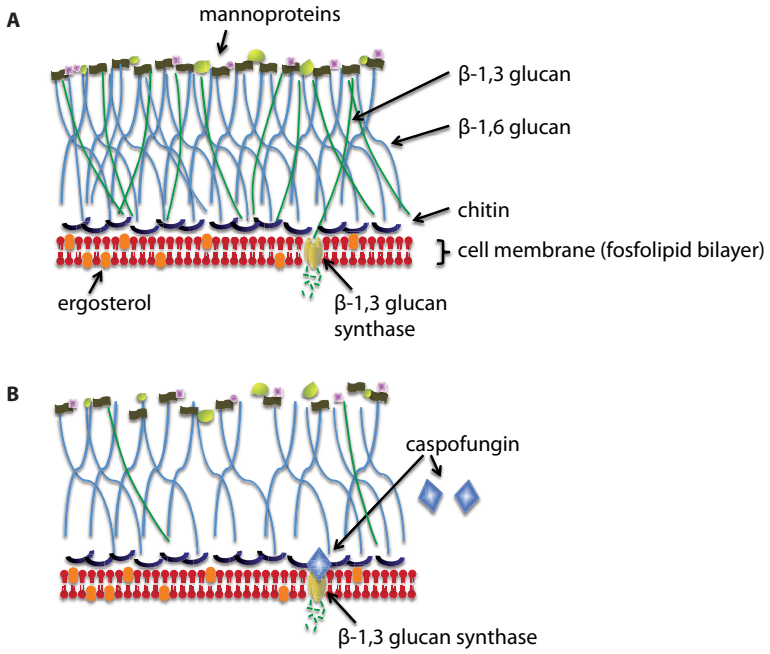

Figure 3. Fungal cell wall (schematic) with its components.
A. Normal fungal cell wall
B. Target and mechanism of action of caspofungin. Caspofungin targets β-1,3 glucan synthase, inhibiting the synthesis of β-1,3 and β-1,6 glucan. This results in a weaker cell wall with less β-glucan.

tions in the growth of *A. fumigatus*, resulting in aberrant morphology with short highly branched hyphae, visible by the naked eye (29). Complete macroscopic growth inhibition of *Aspergillus* species is only observed at high concentrations of caspofungin (29, 30). Based on these *in vitro* results, one would not expect good clinical outcomes after caspofungin therapy. However when patients are treated with relatively low doses of caspofungin, a clinical response is seen. Since the drug itself was not able to inhibit the growth of *A. fumigatus* but only to alter it, it seems that there is a discrepancy between the *in vitro* results of susceptibility testing and the *in vivo* results of treatment with caspofungin. Already years ago, it was shown that human sera, human monocytes and monocyte-derived macrophages enhanced the antifungal activity of caspofungin (26, 31, 32). However, the exact mechanisms behind the clinical response with caspofungin therapy are not well understood and various hypotheses are available.

The studies summarized in this thesis aim to answer the following research question:
How can the discrepancy be explained between the favorable clinical response to treatment with caspofungin, while the *in vitro* antifungal activity of this agent is poor?
To answer this research question, more insight is needed in the mode of action of caspofungin, why it alters *A. fumigatus* growth and the resulting effects on the immune system of the host. We therefore divided this question into several narrower questions and we also investigate new diagnostic and therapeutic strategies for IPA:

- How does caspofungin alter the composition of the fungal cell wall? Is chitin contents upregulated as a result of β-glucan depletion?
- Does altered cell wall composition result in altered immune response of the host? Is cytokine and chemokine response differentially activated after treatment with caspofungin?
- Are chitinases upregulated in IPA as a result of exposure to chitin of the fungal cell wall? Does treatment with caspofungin alter the chitinase response?
- Do genetic polymorphisms, resulting in absent chitinase activity, increase the risk for IPA or for other fungal infections?
- Can newer diagnostic methods successfully identify *A. fumigatus* and distinguish it from morphologically identical species such as *Aspergillus lentulus*?
- Do new agents such as branched histidine and lysin rich peptides have antifungal activity against *A. fumigatus* or yeasts?

EFFECTS OF CASPOFUNGIN ON THE FUNGAL CELL WALL

Chitin and β-glucan are the two main components of the fungal cell wall that are responsible for maintaining its functional strength. As described above, caspofungin inhibits β-1,3-glucan synthase (26, 33, 34), causing a significant inhibition of the synthesis of β-1,3-glucan and β-1,6-glucan. This inhibition results in morphological alterations in the growth in several fungal species (26, 35, 36). In order to understand the biological processes behind this altered morphology, the cell wall components of *Candida* species were analyzed, which showed paradoxical growth at concentrations higher than the MIC of caspofungin (36-38). In several previous publications it was reported that inhibition of the synthesis of β-glucan resulted in upregulation of the chitin synthesis (35-37, 39-46). When *Candida* species were exposed to a combination of a glucan synthase inhibitor and a chitin synthase inhibitor, synergy was noted and fungal growth was significantly decreased (39, 47). In *Aspergillus* species morphological changes were also observed after treatment with caspofungin, and β-glucan synthesis inhibitors and chitin synthase inhibitors were previously shown to act synergistically (30, 39, 43, 44,

46-50). We hypothesized that in filamentous fungi such as *A. fumigatus*, similar changes in cell wall composition could be found as in *Candida* species. Several studies showed that in *A. fumigatus* the β–glucan content is decreased after exposure to caspofungin (26, 44, 46, 48). A compensatory increase in chitin was reported in *A. fumigatus* as well (40, 48). To confirm this finding and to determine if in *A. fumigatus* inhibition of β -glucan synthesis results in an altered chitin synthesis, we investigated the synergy between inhibitors of the β-glucan and chitin synthesis pathways in **Chapter 2**. We combined the β-glucan synthesis inhibitor caspofungin with the chitin synthesis inhibitor nikkomycin Z and studied the morphology and the metabolic activity of *A. fumigatus* after exposure to several combinations of these agents.

EFFECTS OF AN ALTERED FUNGAL CELL WALL COMPOSITION ON THE HOST IMMUNE SYSTEM

Since it was already demonstrated that caspofungin alters the cell wall composition of fungal species, potential effect of this altered cell wall composition on the immune response of the host, needs further investigation. Chitin and β-glucan are molecules on the outer surface of the fungal cell wall and belong to the so-called pathogen-associated molecular patterns (PAMPs), molecules that can be recognized by specific receptors of the host's immune system, pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) (12, 51-53). *A. fumigatus* hyphae are known to be recognized by TLR4 (12). β-glucan is recognized by dectin-1 (53-56) whereas no specific PRR has yet been identified that recognizes chitin (53, 54, 57).

If *A. fumigatus*' cell wall composition is indeed changed by exposure to caspofungin and the level of cell wall chitin is upregulated, this could result in an altered interaction with certain host PRRs and, subsequently, in an altered host immune response. When PAMPs are changed, different PRRs and molecular pathways are activated and signaling cascades are changed (51). This could lead to an increased or decreased production of certain cytokines and chemokines, ultimately influencing the clearance of the fungus. Since it is currently not known which cytokines and chemokines are involved in this process, we investigated the effects of treatment with caspofungin on the levels of pro- and anti-inflammatory cytokines during experimental IPA. We measured levels of 24 cytokines and chemokines in rats with IPA that were either left untreated or that were treated with caspofungin in **Chapter 3**.

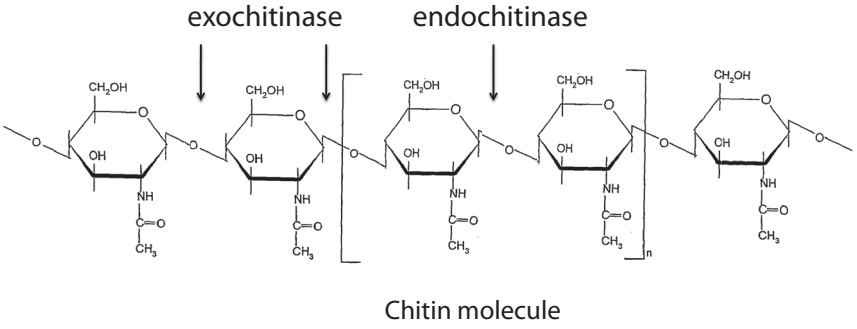

Figure 4. Chitin, chitinases and their sites of action.

CHITINASES, SPECIFIC ENZYMES OF THE HOST IMMUNE SYSTEM, REACTING ON CHITIN

In addition to being recognized as a PAMP, chitin itself is also recognized and cleaved by chitinases, enzymes produced by the host that specifically cleave chitin. Chitinases were first discovered in chitin-containing insects, where they are involved in remodeling their chitin skeleton. Also plants and later mammals were shown to produce these enzymes as defense mechanism against chitin-containing pathogens (58-60). Chitinases are categorized as endochitinase or exochitinase, based upon the site where the hydrolytic activity takes place (Figure 4) (61, 62). Two chitinases have been discovered in humans so far: ***chitotriosidase***, which is an endochitinase and is found in lungs and lacrimal glands of mammals (63-67) and acidic mammalian chitinase (***AMCase***), which is an exochitinase and is found mainly in the gastro-intestinal tract (59). Chitinases are associated with allergic diseases (68, 69), however their role in infectious diseases by chitin-containing fungi was not investigated extensively.

Overdijk *et al* reported increased levels of chitinases in guinea pigs suffering from systemic *A. fumigatus* infections (70, 71). As described in the previous paragraph, we expected the chitin contents of the fungal cell wall to be increased after treatment with caspofungin, resulting in an increased chitinase activity in the host (compared to untreated rats with IPA). This might be responsible for the good clinical response to treatment with caspofungin. In order to investigate the effects of caspofungin therapy on the levels of chitinases, we measured chitotriosidase and AMCase levels in our IPA rat model with IPA in **Chapter 4**.

GENETIC DIFFERENCES IN GENES ENCODING FOR CHITINASES

Previous studies in different geographic regions show that impaired (or decreased) chitinase activity in humans is quite common. Polymorphisms in both chitotriosidase and AMCase have been described, resulting in either increased or decreased enzyme activity (58, 72-75). If indeed chitinase activity plays a role in the defense against fungal infections, the question arises if fungal infections occur more often in individuals with impaired chitinase activity. In other words, we hypothesized that a polymorphism in the genes for chitinases, resulting in impaired chitinase activity, increases the risk for IPA. In addition, increased chitinase activity might also limit the risk for IPA. To test this hypothesis, we investigated whether chitinase impairing polymorphisms are found more often in patients with IPA, compared to patients without IPA. **Chapter 5** describes the prevalence of four different polymorphisms resulting in increased or decreased chitotriosidase or AMCase activity among patients at risk for IPA.

In case the prevalence of these polymorphisms is different in IPA patients compared to patients without IPA, one would hypothesize that this would also be true for infections caused by other fungi. In patients infected with other fungi containing chitin and β-glucan in their cell wall, increased chitinase production by the host could also be induced. In **Chapter 6** we investigated whether the same polymorphisms in the genes for chitinases play a role in the incidence of the tropical disease mycetoma caused by *Madurella mycetomatis* in Sudanese patients.

NOVEL DIAGNOSTIC AND THERAPEUTIC OPTIONS FOR FUNGAL INFECTIONS

As described above, the diagnosis and treatment of fungal infections remain a challenge to clinicians. Next to better understanding of the predictive value of *in vitro* antifungal susceptibility testing for clinical treatment outcome, further improvement of the diagnostic methods and novel therapeutic approaches are of high importance. The last two chapters of this thesis cover novel diagnostic and therapeutic options for fungal infections.

In **Chapter 7** we investigated novel diagnostic techniques to correctly identify *Aspergillus fumigatus* and to distinguish this species from *Aspergillus lentulus*. To distinguish between these two species of *Aspergilli* which are morphologically very similar (76) is important as their susceptibility to antifungal agents is different (77-79). Rapid and correct identification of the causative agent of fungal infections is important in order to enable appropriate treatment at an early stage, leading to better clinical outcomes and to limitation of costs of hospitalization (80).

To identify several *Aspergillus* species including *A. lentulus* and *A. fumigatus*, we compared two relatively new techniques, being Raman spectroscopy and matrix-associated laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS). Both techniques identify pathogens based upon their biophysical properties. In addition to correct identification of *A. fumigatus* and *A. lentulus*, the time frame in which both fungal species can be identified by these two methods is also discussed.

Apart from new diagnostic options, in **Chapter 8** we also investigated new therapeutic strategies, which is important to achieve limitations of costs and toxic side effects of the antifungal drugs currently in use. We investigated a group of agents called branched histidine and lysine-rich peptides (BHKPs). BHKPs were originally developed for gene delivery (81). These peptides were found to have antimicrobial activity and are thought to act in a similar way as histatin-5 (82). Antifungal activity of BHKPs against *Candida* species has been detected, but the antifungal activity against *Aspergillus* species is unknown (82).

Chapter 9 is the summarizing discussion of the main findings presented and discussed in this thesis. It focused on a better understanding of the mechanism of action of caspofungin in IPA, and the role played by host-derived chitinases during these interactions. Furthermore, the results obtained with new antifungal agents are discussed, as well as findings obtained with improved diagnostic methods.

The knowledge gained leads to improved diagnosis and treatment of IPA with either combination therapy of caspofungin, or with newer antifungal agents that target different components of the fungal cell wall. We hope that our results will contribute to novel strategies, resulting in a better clinical outcome.

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CHAPTER 2

Reshuffling of *Aspergillus fumigatus* cell wall components chitin and β-glucan under the influence of caspofungin or nikkomycin Z alone or in combination

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Adapted from Antimicrob Agents Chemother 2012;56(3):1595-1598

ABSTRACT

Chitin and β-glucan are major cell wall components of *Aspergillus* species. We investigated the antifungal activity of chitin synthesis inhibitors nikkomycin Z, polyoxin D, flufenoxuron, lufenuron and teflubenzuron, alone and combined with the β-glucan synthesis inhibitor caspofungin. Only nikkomycin Z and caspofungin were found to act synergistically. The nikkomycin Z-induced chitin decrease corresponded with a β-glucan increase, while with the caspofungin-induced β-glucan decrease, an increase in chitin was found. This could explain the synergistic activity of this combination of drugs.

INTRODUCTION

Chitin and β-glucan, major constituents of the fungal cell wall and not found in humans, are interesting targets for new antifungal strategies. Inhibitors of chitin synthesis have been classified as either peptidyl nucleosides or acylureas. The peptidyl nucleosides, such as nikkomycins and polyoxins, function as substrate analogues and inhibit chitin synthase at its catalytic site (1). The exact mechanism of the acylurea compounds such as teflubenzuron, lufenuron and flufenoxuron is unknown (2).

β-Glucan synthesis is inhibited by the echinocandins, such as caspofungin, which are cyclic lipopeptide compounds inhibiting the enzyme 1,3-β-D-glucan synthase. Lack of chitin and β-glucan in the cell wall leads to osmotic lysis of the fungal cell (1). Therefore, combining a chitin and β-glucan inhibitor may enhance fungal killing.

This study reports the *in vitro* susceptibility of *Aspergillus fumigatus* to different classes of chitin synthesis inhibitors used alone and combined to caspofungin, a β-glucan synthesis inhibitor.

MATERIALS & METHODS

Minimal inhibitory concentrations (MICs) of caspofungin (Merck and Co, Rahway, NJ, USA), and for nikkomycin Z, flufenoxuron, lufenuron, teflubenzuron (all from Sigma-Aldrich, Zwijndrecht, the Netherlands) and polyoxin D (Kaken Pharmaceutical Co., Tokyo, Japan) were determined for 10 *A. fumigatus* strains, including *A. fumigatus* ATCC 204305. For caspofungin, the minimal effective concentration (MEC) of caspofungin was also determined. Nikkomycin Z and polyoxin D were dissolved in water and all other agents were dissolved in dimethyl sulfoxide (DMSO). MICs and MECs were determined in triplicate in RPMI 1640 medium, according to the CLSI broth microdilution method (3).

Synergy between the antifungal agents was investigated by a checkerboard antifungal susceptibility assay. The fractional inhibitory concentration index (FICI) was calculated using method 1 according to Bonapace *et al* (4) with the following formula:

$$FICI = [(MIC_A \text{ in combination})/MIC_A] + [(MIC_B \text{ in combination})/MIC_B]$$

Drug interactions were classified as synergistic ($FICI \leq 0.5$), indifferent ($0.5 < FICI < 4$), or antagonistic ($FICI \geq 4$). For each isolate, FICIs were determined in triplicate.

To assess antifungal activity with respect to fungal metabolic activity, the colorimetric 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt hydroxide (XTT) assay was used (5). Morphology was studied using light microscopy. Concentrations of chitin and β-glucan were determined as previously published (6-9).

RESULTS

Caspofungin was the only agent that inhibited growth of *A. fumigatus* (median MICs and median MEC for 10 strains are shown in Table 1). These MICs were in agreement with previously described MICs for *A. fumigatus* (3). None of the chitin synthesis inhibitors inhibited growth of *A. fumigatus* (Table 1). Microscopic evaluation revealed some morphological alterations after exposure to nikkomycin Z at ≥8 mg/L: some cells were rounded and swollen (Figure 1), though most cells appeared normal.

Table 1. Minimal inhibitory concentrations (MICs) for several groups of antifungal agents. FICIs were calculated in order to assess synergy (see text for calculation method)

Class and antifungal agent	MIC and MEC (μg/ml) (range)*	FICI in combination with caspofungin#
β-glucan synthesis inhibitors		
Echinocandins		
Caspofungin	128 (128 ->128) 0.25 (0.06 – 0.5)*	
Chitin synthesis inhibitors		
Peptidyl nucleosides		
Nikkomycin Z	>64 (>64)	0.15
Polyoxin D	>64 (>64)	2.0
Acylureas		
Flufenoxuron	>16 (>16)	2.0
Lufenuron	>16 (>16)	2.0
Teflubenzuron	>16 (>16)	2.0

* Minimal effective concentration (MEC; indicated by the asterisk) was determined for caspofungin only.
# Median fractional inhibitory concentration index (FICI) of caspofungin in combination with various chitin synthesis inhibitors for 10 *A. fumigatus* strains.

Nikkomycin Z combined with caspofungin resulted in synergy (median FICI of 0.15 for 10 strains), which was not observed with other chitin synthesis inhibitors (median FICI of 2.00; Table 1). Synergy between caspofungin and nikkomycin Z is in agreement with previously published data (1).

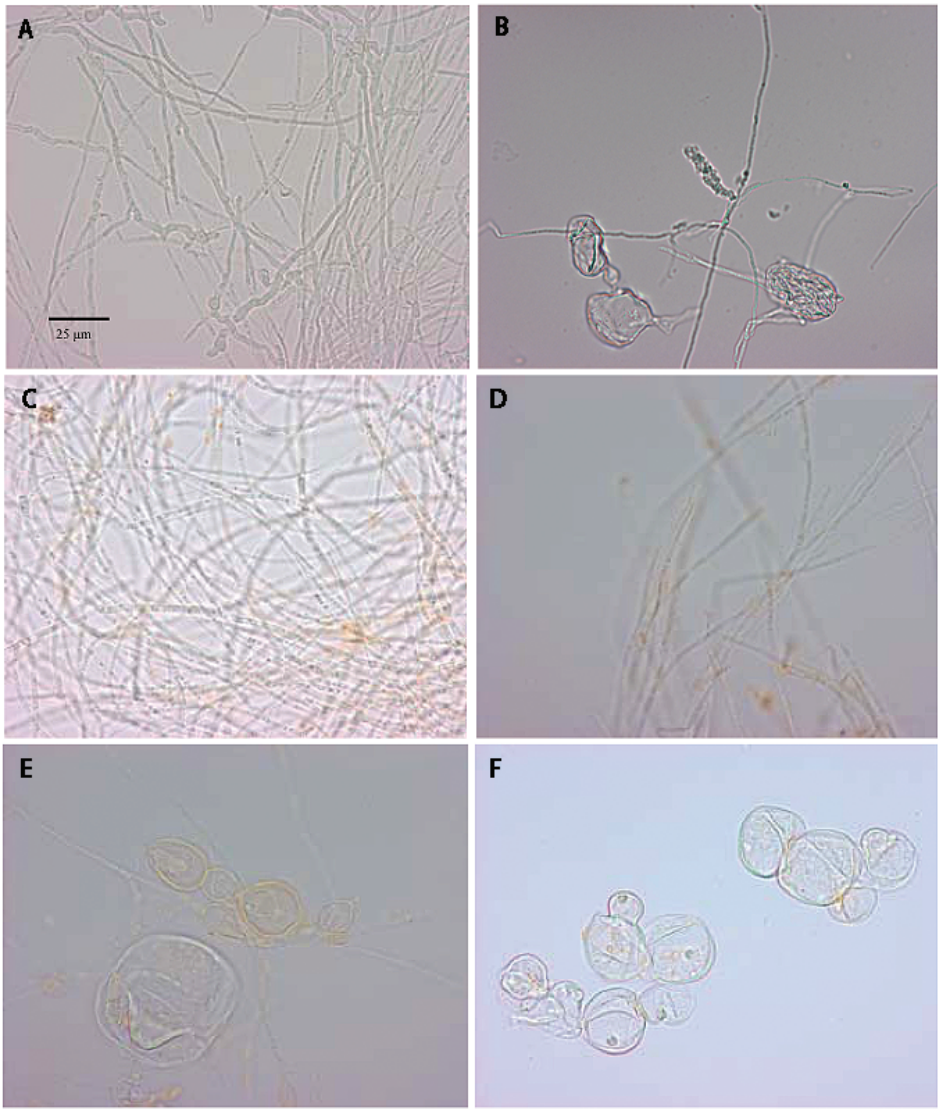

Figure 1. Cells of *A. fumigatus* (original magnification, ×400). Scale bar 25 µm.
A. *A. fumigatus* not exposed to any antifungal agent. Elongated hyphae are noted; no swollen cells are present.
B. *A. fumigatus* exposed to 8 mg/L nikkomycin Z. Elongated hyphae are still seen, but some of the hyphae appear to be swollen and rounded.
C. *A. fumigatus* exposed to 0.125 mg/L caspofungin. Hyphae are shortened, stubby and broad based.
D. *A. fumigatus* exposed to 4 mg/L caspofungin. Hyphae are further shortened, stubby and broad based. This morphology is more evident than with 0.125 mg/L caspofungin.
E. *A. fumigatus* exposed to 0.125 mg/L caspofungin and 8 mg/L nikkomycin Z. Cell morphology is disrupted, and many cells are swollen and rounded, though normal hyphae are still seen.
F. *A. fumigatus* exposed to 4 mg/L caspofungin and 8 mg/L nikkomycin Z. Cell morphology is completely disturbed and no normal hyphae are seen.

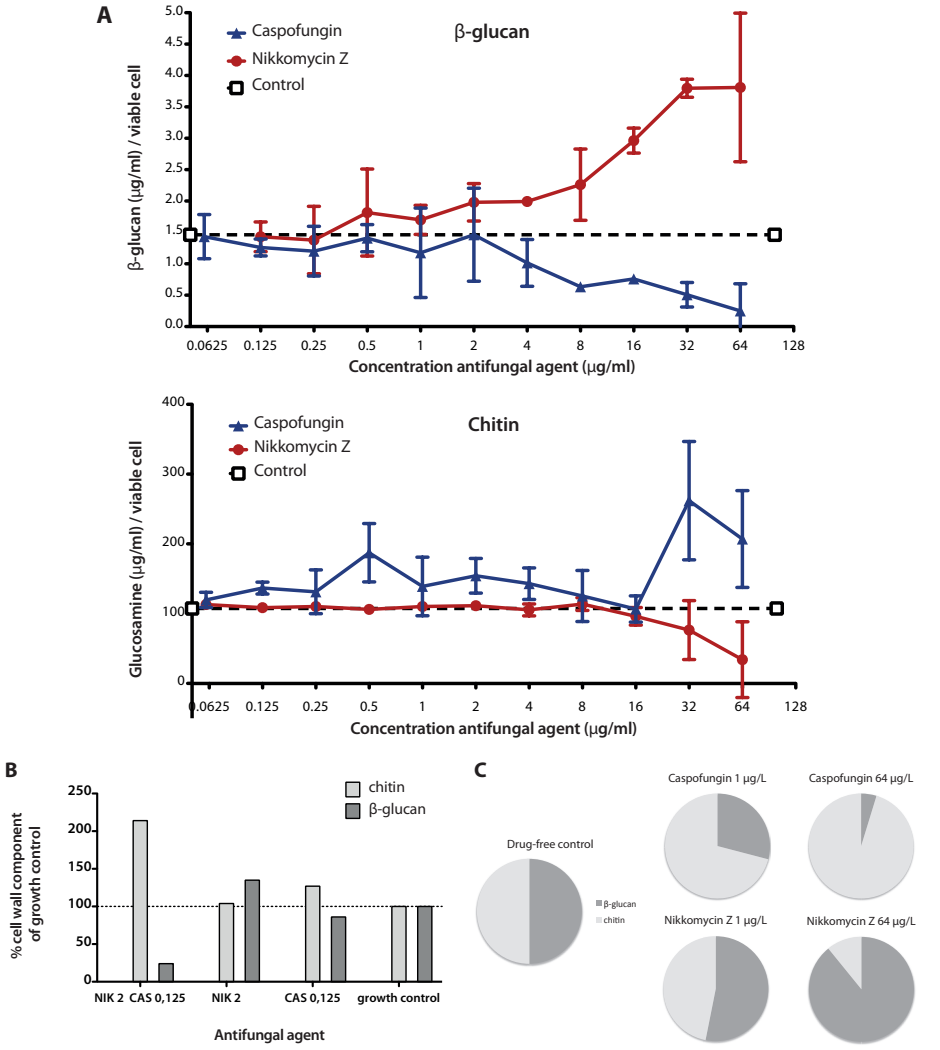

Figure 2. Concentrations of the cell wall components β-glucan and chitin in *A. fumigatus* ATCC 204305 after exposure to caspofungin and nikkomycin Z at various concentrations.

A. Glucosamine concentrations represent chitin concentrations. Means from three independent experiments are shown. Bars represent standard deviations. β-Glucan and chitin concentrations were corrected to the number of viable cells with the XTT assay by the following formula: (amount of β-glucan or chitin measured) × (number of viable cells in the growth control/number of vibale cells in the well tested).

B. Fungal response in cell wall components. Concentrations of β-glucan and chitin after expoure to 0.125 µg/ml of caspofungin combined with 2 µg/ml nikkomycin Z, each agent alone, and the growth control. This graph shows that combining caspofungin and nikkomycin Z causes a drastic decrease in β-glucan content, resulting in a compensatory increase in chitin content.

C. Shift in cell wall components β-glucan and chitin after exposure to caspofungin and nikkomycin Z at two concentrations (schematic representation). Both components are standardized to 50% in the drug-free control.

Antifungal activity with respect to fungal metabolic activity was tested using the colorimetric XTT assay (5). Exposure to none of the agents alone led to >80% decreased metabolic activity of the fungal cells, except for caspofungin at 128 mg/L. Similar results were described by Antachopoulos *et al* (10). However, combining caspofungin and nikkomycin Z mostly affected the decrease in fungal metabolic activity. For example, 0.125 mg/L caspofungin alone resulted in an 8% decrease in metabolic activity, whereas 0.125 mg/L caspofungin combined with 2 mg/L nikkomycin Z resulted in a 56% decrease in metabolic activity (data not shown). The morphology of fungal cells exposed to the drugs in combination was also studied (Figure 1). This figure shows that when *A. fumigatus* was exposed to 8 mg/L nikkomycin Z, elongated hyphae were still present but some of the cells became rounded (Figure 1B). Exposure to caspofungin caused short, stubby branched hyphae (Figure 1C and D). When both agents were combined, the rounded cells became more prevalent (Figure 1E). At a concentration of 4 mg/L caspofungin combined with 8 mg/L nikkomycin Z, the cell morphology was completely disturbed and no hyphae were identified anymore (Figure 1F).

To elucidate the observed synergy between caspofungin and nikkomycin Z, 5×10^4 conidia/ml were exposed to these drugs for 48 hours and the changes in cell wall components chitin and β-glucan were determined. At caspofungin concentrations of 4 mg/L and greater, a decrease in β-glucan content was observed (Figure 2A). Additionally, with caspofungin concentrations of 32 mg/L and greater, chitin concentrations were elevated (Figure 2B). With nikkomycin Z, the opposite was observed: β-glucan concentrations increased when a concentration of 0.5 mg/L or greater was used, while chitin concentrations decreased when a concentration of 16 mg/L or greater was used. This observation is in agreement with Fortwendel *et al*, who reported that exposure to nikkomycin Z in concentrations up to 16 mg/L did not influence chitin concentrations (11). The combination of nikkomycin Z and caspofungin caused instability of the fungal cell wall. Figure 2C shows that β-glucan concentrations were strongly decreased after exposure to 0.125 mg/L caspofungin combined with 2 mg/L nikkomycin Z. As a consequence, chitin concentrations were increased in order to survive. This effect was also found for other combinations (data not shown).

DISCUSSION

Exposure to a single drug, either caspofungin or nikkomycin Z, causes a reshuffling of cell wall components chitin and β-glucan (schematically shown in Figure 2D). Inhibition of chitin synthesis in *A. fumigatus* resulted in increased synthesis of β-glucan and vice versa. This would explain the synergy between both agents as well as the altered

morphology after exposure to caspofungin and nikkomycin Z. This alteration in cell wall components is not unique for *A. fumigatus*. Stevens *et al* showed that *Candida albicans* reacts to caspofungin concentrations above the MIC by compensatory increases of the chitin contents of the cell wall, resulting in survival (12). Furthermore, Walker *et al* reviewed several studies that investigated the enhancement of echinocandins activity when combined to nikkomycin Z in yeasts and fungi (13).

Nikkomycin Z was the only chitin synthesis inhibitor that inhibited *A. fumigatus* growth when combined to caspofungin. Since polyoxin D is also a peptidyl nucleoside, we expected to find the same synergy. However, polyoxin D, either alone or combined with caspofungin, did not inhibit growth of *A. fumigatus*. Polyoxin D is capable of inhibiting growth of *Saccharomyces cerevisiae* (14, 15), *C. albicans* and *Cryptococcus neoformans* (16). Archer showed that polyoxin D does inhibit *A. fumigatus* chitin synthase (17). However, in the present study, the inhibition of chitin synthase by polyoxin D, even when combined with caspofungin, was not sufficient to result in fungal growth arrest.

In summary, we have shown that the inhibition of the synthesis of a single cell wall component (either β-glucan or chitin) results in a subsequent increase in synthesis of the other cell wall component. The observed synergy between caspofungin and nikkomycin Z is probably the result of an inability of the fungus to compensate for the altered ratio of chitin to β-glucan. Indeed, Luque *et al* showed that the β-glucan synthesis inhibitor micafungin combined to the chitin synthase inhibitor nikkomycin Z improved survival in mice with systemic aspergillosis (18). Additional studies are needed to investigate the *in vivo* potential of nikkomycin Z and caspofungin as a new antifungal combination for the treatment of aspergillosis.

ACKNOWLEDGEMENTS

The authors would like to thank Kaken Pharmaceutical Co., Ltd. for kindly providing the polyoxin D.

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CHAPTER 3

Effect of caspofungin on cytokine kinetics in experimental invasive pulmonary aspergillosis – A descriptive study

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Manuscript submitted

ABSTRACT

Caspofungin is effective against invasive pulmonary aspergillosis (IPA) in immunocompromised hosts. *In vitro* however, caspofungin has no detectable fungicidal or fungistatic activity on hyphal growth in *Aspergillus fumigatus*, although aberrant growth is observed. We hypothesized that the *in vivo* beneficial effects of caspofungin may be the result of a caspofungin-induced change in immune response, thus improving patients' outcomes.

To investigate our hypothesis, we determined levels of cytokines and chemokines in an experimental model of lethal IPA in neutropenic rats. Neutropenic rats were infected intratracheally with *A. fumigatus* conidia. Levels of 24 pro- and anti-inflammatory cytokines and chemokines were measured at several intervals in different body compartments to determine the effects of caspofungin treatment on host immune response relative to survival.

IPA induced changes in the level of many cytokines, the response in the infected lungs being different from that in the blood compartment. In lungs, IL-6, IP-10, KC (equivalent of human IL-8) and MCP-1 increased at least 100-fold or more. In serum, GM-CSF, IL-12p70, IL-10 and KC likewise increased. Life-saving treatment with caspofungin (achieving 100% rat survival) resulted in a similar immune response, though the cytokine response in the lungs, especially of MCP-1, was attenuated.

In conclusion, in an experimental rat model of lethal IPA in neutropenic rats, life-saving caspofungin treatment was associated with attenuation of the hosts' proinflammatory response in the lung, however, levels of cytokines were similar in caspofungin-treated rats as in rats left untreated.

INTRODUCTION

Over the last decades, the incidence of *Aspergillus* species infections is increasing (1). In immunocompromised patients, invasive pulmonary aspergillosis (IPA) remains a cause of severe mortality and morbidity. Currently the first choice therapy consists of voriconazole.

Caspofungin, belonging to the class of echinocandins, is used as salvage therapy for invasive aspergillosis (2). Caspofungin inhibits the β-glucan synthesis and, therefore, has a direct effect on the composition of the *Aspergillus* cell wall. *In vitro* exposure to caspofungin results in an aberrant hyphal growth of *Aspergillus fumigatus*, but not in growth inhibition or death of the fungus, which is commonly observed for other antifungal agents. In contrast, *in vivo*, in our model of IPA in transiently neutropenic rats, which has a fatal course if left untreated, the human equivalent dosage of caspofungin resulted in 100% rat survival (3). Therefore the question arises what causes this excellent therapeutic outcome, as caspofungin itself has no apparent activity on the viability of the fungal mycelium.

When *A. fumigatus* is exposed to caspofungin, β-glucan synthesis is inhibited and the chitin component of the cell wall is upregulated *in vitro* (4). Both chitin and β-glucan function as pathogen-associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs) of the hosts' immune system. Since caspofungin alters the fungal cell wall composition, the type and amount of PAMPs are also changed (5). The alteration of fungal PAMPs is known to result in an altered immune response (6) and thus we hypothesized that an altered cytokine response was responsible for the beneficial clinical response to caspofungin in *Aspergillus* infections.

No studies were published with respect to the cytokine profile of neutropenic patients with IPA. We studied the levels of 24 pro- and anti-inflammatory cytokines and chemokines in our IPA model in neutropenic rats treated with caspofungin versus rats left untreated.

MATERIALS & METHODS

Experimental animal model

The model of invasive pulmonary aspergillosis (IPA) in neutropenic rats was previously described in detail (3, 7). Some minor changes have led to the following experimental design. Female strain RP/AEur/RijHsd albino rats were used in all experiments. Animals 18-25 weeks of age, body weight 200-250 g with specified pathogen-free status were used. Transient neutropenia was induced by intraperitoneally (i.p.) administration of

cyclophosphamide (Endoxan, Baxter, Utrecht, The Netherlands) in doses of 75, 60, 50 and 40 mg/kg at day 5 and day 1 before fungal inoculation, and at day 3 and day 7 after fungal inoculation, respectively. This protocol resulted in substantially decreased leukocyte and granulocyte counts (7). To prevent bacterial superinfections, rats were given ciprofloxacin (500 mg/L) and colistin (100 mg/L) in their drinking water ad libitum. Furthermore, rats were given teicoplanin intramuscularly in doses of 30 mg/kg on days 5 and 1 before fungal inoculation, and 15 mg/kg on days 1, 3, 6, 8 and 10 after fungal inoculation.

A clinical *A. fumigatus* isolate was used for infection. This strain was originally isolated from a hemato-oncological patient with IPA. Left-sided pulmonary infection was established by intubation of the left main bronchus, while the rats were under general anesthesia. A cannula was passed through the tube and the left lung was inoculated with 20 µl phosphate buffered saline containing 6×10^4 conidia of *A. fumigatus*. Sham-infected animals were inoculated with 20 µl saline. Caspofungin–treated rats received saline-diluted caspofungin (Merck & Company, Rahway, NJ, USA) i.p. once daily in a dose of 4 mg/kg/day, starting at 24h after initiation of the infection. Untreated animals received saline i.p. Regarding the experimental set-up, the experiment consisted of 4 groups of rats: A) infected, untreated rats; B) infected, caspofungin-treated rats; C) sham-infected, untreated rats and D) sham-infected, caspofungin-treated rats.

Blood samples were taken from the tail vein on day 1, 3 or 6 after initiation of infection and serum was isolated and stored at −80°C within 2 hours after sampling. Next, rats were sacrificed by CO_2 exposure. Lungs were dissected at the same time points and were either stored at −80°C until further processing, or fixated in formalin to be used for immunohistochemistry. Tissue coupes were stained with Grocott methenamine-silver. On each time point, all groups consisted of 5 rats, except the group of infected rats left untreated on day 6, which consisted of 4 rats as 1 out of 5 rats died. Infected organs and blood from rats found dead were always cultured to exclude bacterial superinfections.

The animal experimental protocols adhered to the rules laid down in the Dutch Animal Experimentation Act and the EU Animal Directive 2010/63/EU. The Institutional Animal Care and Used Committee of the Erasmus University Medical Centre Rotterdam approved the present protocols.

Determination of cytokine levels

Lungs were homogenized and cytokine levels were determined in both lung homogenate and serum using a bead-based flow cytometry technique (xMap; Luminex Corporation, Austin, TX, USA). The rat soluble cytokine receptor multiplex panel that was used (BioLegend custom Rat LEGENDPlex 24) contained bead-labelled cytokine

receptors against pro- inflammatory cytokines (granulocyte-colony stimulating factor [G-CSF], granulocyte macrophage-stimulating factor [GM-CSF], interferon [IFN-γ], interleukin [IL]-1α, IL-1β, IL-2, IL-6, IL-12p70, IL-17a, IL-18, IL-21, IL-22, tumour necrosis factor [TNF-α] and thymic stromal lymphopoietin [TSLP]), anti-inflammatory cytokines (IL-4, IL-5, IL-10 and IL-13), chemokines (interferon-gamma induced protein [IP]-10, keratinocyt chemoattractant [KC, the murine functional homologue of human IL-8], monocyte chemoattractant protein [MCP]-1, macrophage inflammatory protein [MIP-1α] and regulated on activation, normal T-cell expressed and secreted (8)) and the regulatory vascular endothelial growth factor (VEGF). Assays were performed according to the manufacturer's instructions and samples were measured in duplicate. Cytokine levels were converted from median fluorescence intensity (MFI) to pg/mL using MILLIPLEX Analyst software (Millipore) and duplicates were averaged.

The immune response in untreated rats is defined by the absolute difference between infected, untreated rats (group A) and sham-infected, untreated rats (group C), which is called $\Delta_{UNTREATED}$. The immune response in caspofungin-treated rats is defined by the absolute difference between infected, caspofungin-treated rats (group B) and sham-infected, caspofungin-treated rats (group D), which is called $\Delta_{TREATED}$. At each time point and for each cytokine or chemokine, $\Delta_{UNTREATED}$ was compared with $\Delta_{TREATED}$ and the difference was assessed for statistical significance. In short:

$\Delta_{UNTREATED}$ = (cytokine level in infected, untreated rats) – (cytokine level in sham-infected, untreated rats)

$\Delta_{TREATED}$ = (cytokine level in infected, treated rats) – (cytokine level in sham-infected, treated rats)

$\Delta_{UNTREATED}$ and $\Delta_{TREATED}$ were compared and tested for significance.

Statistics

Absolute cytokine levels of infected rats were compared with cytokine levels of sham-infected rats using Mann-Whitney U-test (GraphPad Prism Software, San Diego, USA). The relative increase or decrease in cytokine level was expressed as an x-fold increase. Median cytokine levels of infected animals were divided by median cytokine levels of sham-infected animals on the same time point, resulting in an x-fold increase of the cytokine level.

The cytokine response in caspofungin-treated rats was compared with the cytokine response in sham-treated rats as follows. Absolute differences in cytokine levels (Δ-cytokine) were calculated between individual infected and sham-infected rats. At each time point, Δ-cytokines were compared between caspofungin-treated rats and sham-treated rats, using Mann-Whitney U-test. A p-value of <0.05 was considered significant.

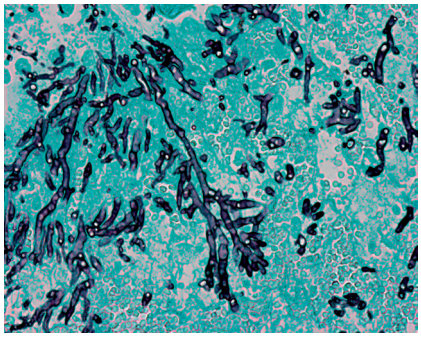

Figure 1. Grocott staining of *Aspergillus fumigatus* infected lungs. Fungal hyphae are stained black. Magnification ×400.

RESULTS

Rats infected with *A. fumigatus* conidia developed invasive pulmonary aspergillosis (IPA), as shown in Figure 1. All untreated rats died within 7 days and all caspofungin-treated rats survived, as we previously described elsewhere (3). To investigate the cytokine kinetics during this infection, pro- and anti-inflammatory cytokine levels were determined in both serum and in homogenized lung, in order to compare cytokine responses at the site of infection with the cytokine levels simultaneously present in the blood compartment.

Pulmonary cytokine levels

IPA caused pulmonary insufficiency and ultimately death in all rats. No pulmonary discomfort or death was observed in sham-infected rats.

The pulmonary cytokine levels of the infected rats differed considerably from those of sham-infected rats. In infected rats, levels of pro-inflammatory IFN-γ, IL-1α, IL-6, IL-18, IL-21, IL-22 and TNF-α and chemokines IP-10, KC, MCP-1, MIP-1α and RANTES increased during the course of infection (Supplementary Table S1). An exception was TSLP, which decreased during infection, although not statistically significant. The changes observed in the levels of other cytokines measured in lung homogenates were statistically not significant (data not shown). For the 12 cytokines and chemokines that changed significantly during the infection, we calculated the relative changes. For IL-6, IP-10, KC and MCP-1 the relative change was most remarkable, since levels of these proteins increased over 100 fold (Figure 2A), with maximum levels reached on day 6 after infection (Figure 3). The median level of IL-6 being 0.14 pg/mL before infection remained unchanged at day 1 after infection and from there increased to 95 and 569 pg/mL on day 3 and day 6 after infection, respectively (Figure 3A). In infected rats, the median level of IP-10 increased from 4.3 pg/mL before infection up to 5.5, 44 and 150 pg/mL on day 1, day 3

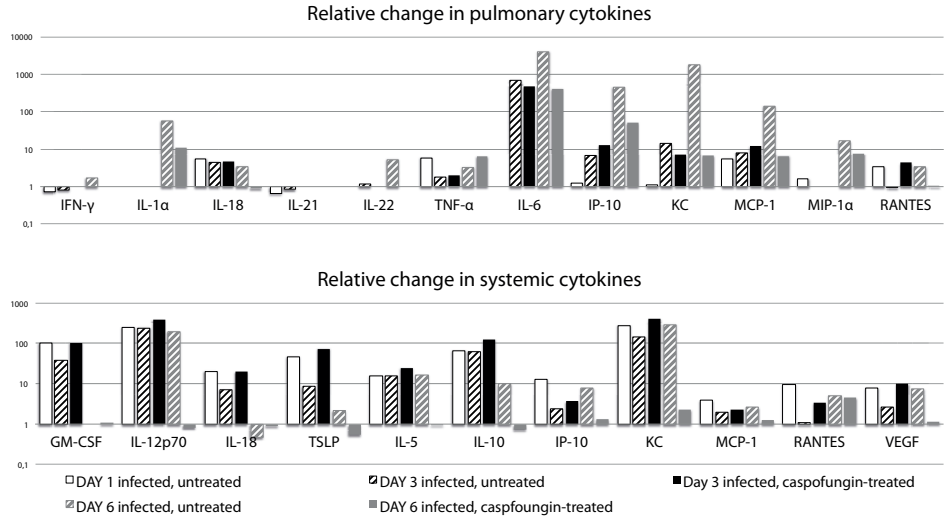

Figure 2. Relative changes in cytokines and chemokines during IPA.
A. Pulmonary cytokines in the lung
B. Systemic cytokines in serum.
Median cytokine levels in infected rats were divided by median cytokine levels of sham-infected rats, resulting in a fold-increase (y-axis). Levels of cytokines in infected, untreated rats (group A) were compared with those in sham-infected, untreated rats (group C). Levels of cytokines in infected, caspofungin-treated rats (group B) were compared with those in sham-infected, caspofungin-treated rats (group D). White bars: infected rats on day 1, before caspofungin treatment was started. Black striped bars: infected rats on day 3. Black bars: infected caspofungin-treated rats on day 3. Grey striped bars: infected rats on day 6. Grey bars: infected caspofungin-treated rats on day 6. Pulmonary IL-6, IP-10, KC and MCP-1 all show a relative increase of 100× or more at any time during IPA. In contrast, systemic GM-CSF, IL-12p70, IL-10 and KC show a relative increase of 100× or more during IPA. Each sample was measured in duplicate and duplicates were averaged. N=5 for each group, except infected rats left untreated on day 6 (n=4).

and day 6 after infection, respectively, whereas in sham-infected rats, median level of IP-10 decreased to 0.33 pg/mL on day 6 after infection (Figure 3B). The median level of KC in infected rats increased from 107 pg/mL before infection to 119, 550 and 8796 pg/mL on day 1, day 3 and day 6 after infection, respectively, whereas in sham-infected rats KC decreased to 5 pg/mL on day 6 after infection (Figure 3C). The median level of MCP-1 increased from 63.4 pg/mL before infection to 351, 818 and 18849 pg/mL on day 1, day 3 and day 6 after infection, respectively (Figure 3D).

Serum cytokine levels

The lung is the primary site of IPA. Cytokines produced in the lung do not always spill over into the systemic circulation (9). Therefore, it cannot be assumed that during IPA the same cytokine profiles develop in the blood compartment as in the lung. Therefore,

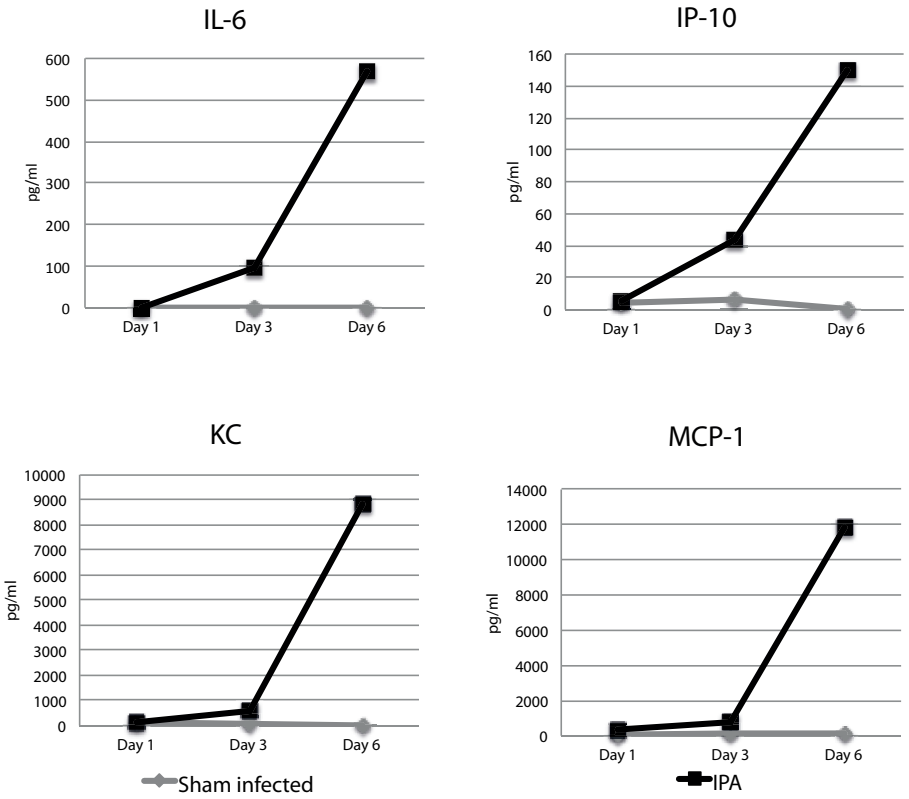

Figure 3. Absolute levels of pulmonary IL-6 (panel A), IP-10 (panel B), KC (panel C) and MCP-1 (panel D) during IPA. Rats were sham-infected or infected with *A. fumigatus* and were left untreated. Median levels of cytokines are shown. Each sample was measured in duplicate and duplicates were averaged. N=5 for each group, except infected rats left untreated on day 6 (n=4).

we also determined cytokine levels in serum samples at the same time points. During infection, 11 out of 24 cytokines and chemokines showed changes. Serum levels of pro-inflammatory GM-CSF, IL-12p70, IL-18 and TSLP, anti-inflammatory IL-5 and IL-10, chemokines IP-10, KC, MCP-1, RANTES and of regulatory VEGF varied significantly during the course of infection (Supplementary Table S2). All of these cytokines increased during infection but some cytokine levels decreased after an initial increase. For all 11 cytokines that changed significantly during the course of infection, we calculated relative changes (Figure 2B). Levels of GM-CSF, IL-10, IL-12p70 and KC increased over 100-fold within 24 hours of infection. The median level of GM-CSF increased from 0.44 pg/mL before infection to 45.2 on day 1 after infection and then decreased to 16.6 pg/mL on day 3 after infection (Figure 4A). On day 6, GM-CSF level returned to levels from prior infection (0.44 pg/mL, Figure 4A). Apparently, GM-CSF is increased in the early stage of the infection

only. IL-10 showed a similar pattern. The median level of IL-10 increased from 0.39 pg/mL before infection to 25.6 pg/mL on day 1 after infection and subsequently decreased to 24.2 and 3.9 pg/mL on day 3 and day 6 after infection, respectively (Figure 4C). Median levels of IL-12p70 also increased early in the infection but did not return to levels from prior to infection. The median level of IL-12p70 increased from 0.18 pg/mL before infection to 43.9 pg/mL on day 1 after infection and then decreased to 34.2 pg/mL on day 3 and day 6 after infection, respectively (Figure 4B). The median level of KC increased from 0.47 pg/mL prior to infection to 127, 66.7 and 138.5 pg/mL on day 1, day 3 and day 6 after infection, respectively (Figure 4D).

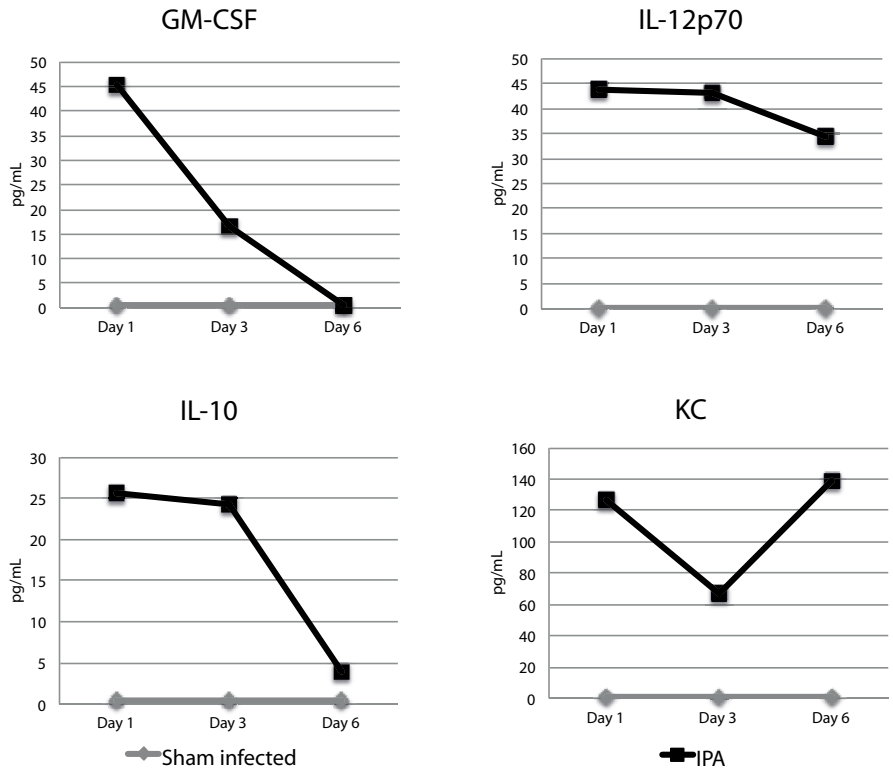

Figure 4. Absolute levels of systemic GM-CSF (panel A), IL-12p70 (panel B), IL-10 (panel C) and KC (panel D) during IPA. Rats were sham-infected or infected with *A. fumigatus* and were left untreated. Median levels of cytokines are shown. Each sample was measured in duplicate and duplicates were averaged. N=5 for each group, except infected rats left untreated on day 6 (n=4).

Pulmonary versus systemic cytokine kinetics

During IPA cytokine kinetics in the lungs were not similar to the cytokine kinetics in serum. We found that pro-inflammatory IL-18 and chemokines IP-10, KC, MCP-1 and

RANTES were significantly increased in both serum and in the lung, however followed a different pattern during infection in each compartment. For example levels of KC in serum show a stronger increase during infection than in the lungs, whereas levels of MCP-1 in serum showed a milder increase during infection than in the lungs (Figure 2A,B).

The remaining cytokines and chemokines were increased in either lung or in serum. Pro-inflammatory IFN-γ, IL-1α, IL-6, IL-21, IL-22, TNF-α and chemokine MIP-1α were increased only in the lungs. In contrast, pro-inflammatory GM-CSF, IL-12p70 and TSLP, anti-inflammatory IL-5 and IL-10 and regulatory VEGF were increased exclusively in serum and not in the lungs. From these data we concluded that kinetics of cytokines differed in each compartment, which is in line with previously published data, showing that systemic levels of cytokines and chemokines do not always correspond with local levels of cytokines (9).

Cytokine kinetics in caspofungin-treated rats

In order to investigate the influence of caspofungin on the cytokine responses in neutropenic rats, we conducted several experiments. To test whether caspofungin itself has any immunomodulatory effects, we measured cytokine levels in sham-infected, caspofungin-treated rats (group D) and compared them with those in sham-infected, untreated rats (group C). We found only minimal non-significant differences between levels of cytokines and we concluded that caspofungin itself, at the dose used, has no immunomodulatory effects.

As described before, caspofungin itself has poor *in vitro* fungicidal activity. However, *in vivo*, patient outcomes improve in response to treatment with caspofungin. In order to test whether the innate immune system plays a role in this paradox, we compared levels of cytokines in infected, untreated rats (group A) with those in infected, caspofungin-treated rats (group B).

IPA itself caused a rise in many cytokines in the lung, as described above. However, treatment with caspofungin did not essentially alter the nature of the cytokine responses, although some attenuation in the cytokine response, especially in the last phase in infection, day 6, seemed to occur in caspofungin-treated hosts (Figure 2A,B).

To further assess the capacity of caspofungin to influence the cytokine response, we compared the immune response in untreated rats (groups A and C) with that in caspofungin-treated rats (groups B and D; for calculations and statistics: see Material & Methods). The Δ-cytokines caused by IPA was compared between untreated rats on the one hand, and caspofungin-treated rats on the other hand. The differences between $\Delta_{UNTREATED}$ and $\Delta_{TREATED}$ thus calculated were significant only for two cytokines in different compartments. The $\Delta_{UNTREATED}$ of IL-22 in the lungs on day 3 after infection was signifi-

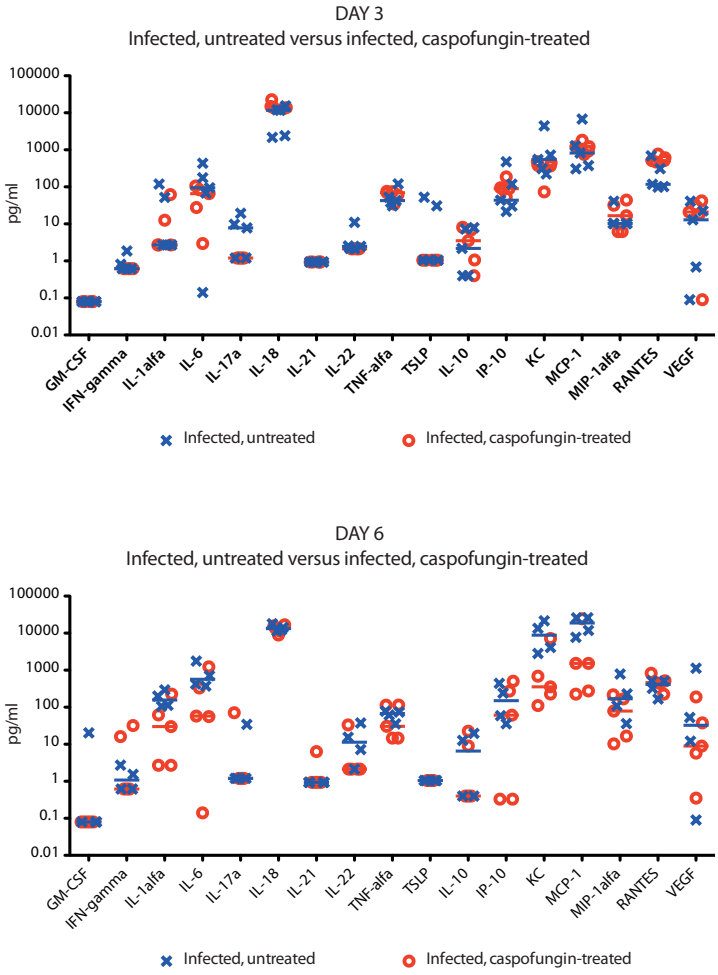

Figure 5. Concentration of cytokines in lungs of infected, untreated rats (blue crosses) and of infected, caspofungin-treated rats (red circles) on day 3 (panel A) and day 6 (panel B) after infection. Bars show medians. Each sample was measured in duplicate and duplicates were averaged. N=5 for each group, except infected rats left untreated on day 6 (n=4).

cantly different from $\Delta_{TREATED}$, however the difference was small (the infection caused an increase of 0.35 pg/ml on day 3 in untreated rats vs. no difference in treated rats, respectively; p=0.047). Furthermore, the $\Delta_{UNTREATED}$ of MCP-1 in serum on day 6 after infection was significantly different from $\Delta_{TREATED}$ (the infection caused an increase of 376 pg/mL on day 6 after infection in untreated rats vs. 59 pg/mL in treated rats, respectively; p=0.032). These results show that in this model, caspofungin leads to attenuation of the increase in MCP-1 in serum on day 6 after infection and, to a lesser extent, of the increase in IL-22 in the lungs on day 3 after infection. The $\Delta_{UNTREATED}$ and $\Delta_{TREATED}$ for all other cytokines

and chemokines at any other moment during infection were not significantly different between caspofungin-treated and untreated rats.

Absolute cytokine levels in infected rats

As shown in the section above, the Δ-cytokine was very similar in caspofungin-treated rats as compared to untreated rats. However, the rat survival in both groups was very different. In addition to evaluating the relative increase in cytokine levels (see previous section), we also assessed absolute cytokine levels. The absolute levels of pulmonary cytokines in infected rats varied considerably from rat to rat within a group of treated or untreated rats on the same day after infection. On day 3 after infection, median levels of cytokines are similar in caspofungin-treated rats versus untreated rats ($p>0.05$; data not shown).

On day 6 after infection, absolute cytokine levels were very similar as well, though some differences can be noted. Median levels of MCP-1 in the lungs were >10× lower in caspofungin-treated rats when compared to untreated rats (18849 pg/mL in untreated rats and 1528 pg/mL in caspofungin-treated rats, $p=0.032$). Median levels of KC in the lungs were 8796 pg/mL in untreated rats and 356 pg/mL in caspofungin-treated rats ($p=0.064$). Although this latter difference did not reach statistical significance, probably due to the small numbers of rats used per group, such difference in cytokine response might be crucial in the survival of the caspofungin-treated rats. Levels of other cytokines in the lungs and levels of all cytokines in serum were in the same range in caspofungin-treated rats compared to untreated rats.

In summary, treatment with caspofungin caused a limitation of the increase in MCP-1 and KC in the lungs that is caused by IPA.

DISCUSSION

IPA remains a disease in the immunocompromised host and always shows a fatal course, despite treatment with antifungal agents. Caspofungin is one of the regularly used antifungal agents, though it is not well understood why patients recover from IPA after treatment in view of the limited *in vitro* susceptibility of *Aspergillus fumigatus* to caspofungin (7). The aim of the present study was to further investigate the mechanism of action of caspofungin and to examine the hypothesis that the hosts' immune system plays a role. We used our earlier developed animal model of IPA in transiently neutropenic rats showing 100% mortality of rats within 6-7 days (10). In a previous study we demonstrated that early treatment with caspofungin in the human pharmacokinetic equivalent dose resulted in 100% rat survival (3, 11). In another study we showed that host's chitinase activity is increased in the lungs of neutropenic rats when inoculated

with *A. fumigatus* (7). As chitinases do not have a direct fungistatic or fungicidal effect, other factors are probably involved in the success of treatment with caspofungin. To determine whether the caspofungin-induced change in cell wall composition resulted in a change in cytokine response to infection, which could be responsible in part for the *in vivo* efficacy of this drug, we measured cytokines in caspofungin-treated rats. To our knowledge, no other studies were published regarding the cytokine profile after treatment with caspofungin.

In the present study we investigated the role of cytokines during the course of IPA in the neutropenic host. The strength of our study is that we investigated a broad panel of 24 cytokines and chemokines. We found that IPA resulted in a rise in the levels of multiple cytokines and chemokines, indicating that the neutropenic host is still able to produce many pro- and anti-inflammatory cytokines. Similar observations for a few cytokines were made by other investigators (12, 13). As IPA is an opportunistic infection that exclusively develops in the immunocompromised host, we performed our experimental studies in immunocompromised rats with neutropenia, induced by cyclophosphamide. This agent is clinically used particularly in hematologic patients, resulting in prolonged neutropenia and thus at risk to develop IPA (http://www.drugs.com/pro/ cyclophosphamide.html).

Several studies investigated the cytokine response in immunocompetent rats with IPA (5, 14-17). In these papers, TNF-α, IL-1α, IL-1β, MIP-1α, MIP-1β, KC, IL-17a, IL-12p40, IL-23, TSLP, IL-10, IFN-γ were found increased after infection with *A. fumigatus*. Conflicting results were reported for IL-4. El Muzghi *et al* found increased levels of IL-4 (14) whereas Urb *et al* found no increase (16). Differences in experimental setup may explain the discrepancy in results.

It should be noted that immunocompromised hosts respond differently to infection than immunocompetent hosts, which was confirmed by Herbst *et al* (17). They showed that cytokine response after exposure to *A. fumigatus* was attenuated in immunocompromised rats compared to immunocompetent rats. In the studies describing cytokine panels in animal models of the immunocompromised host, different methods were used to induce immunosuppression, including corticosteroid treatment, neutrophil neutralizing antibodies and cyclophosphamide (12, 13, 17-20). Cytokines and chemokines were measured in several compartments, including lung homogenate, broncho-alveolar lavage (BAL) fluid and culture supernatants in which spleen cells were incubated. These studies performed in different models of IPA showed that levels of TNF-α, IFN-γ, IL-1β, IL-12p70, MIP-2, KC and IL-10 were all increased. Our results are partly in agreement with these findings. We found no increase in IFN-γ and in IL-1β. However, for TNF-a, IL-12p70, KC and IL-10 we did find increased levels occasionally during the infection. MIP-2 levels

were not investigated in our study. In other infections, including scrub typhus in humans and severe *Staphylococcus aureus* bacteremia in mice, it was shown that increased levels of IL-8 or KC, the murine equivalent of human IL-8, were associated with disease severity or fatal outcome (21, 22). Even though KC levels did not reach statistical significance in our study, we observed a trend for a similar correlation.

As IPA presents heterogeneously in patients at risk for IPA, it is difficult to interpret cytokine responses in these patients. Data obtained from a well-defined experimental animal model as used in the present study may provide further insight into changes in the cytokine profile during the course of IPA. However, it should be noted that cytokine responses in rodents differ, at least in some aspects, from cytokine responses in humans. For example, we found no increase in IL-17 in rats as a result of IPA. Both Werner *et al* and El Muzghi *et al* showed that IL-17 was produced in pulmonary aspergillosis in non-neutropenic mice (14, 23) though production of IL-17 was not induced in human peripheral blood mononuclear cells after exposure to live *A. fumigatus* conidia or in patients with probable or proven aspergillosis (24). Thus, extrapolation of the present data to the human situation should be done with caution. In addition, IL-17 and the IL-17 receptor were shown to be of major importance in neutrophilic inflammation of the airways (25). In our study we found no increase in IL-17. In our model rats are neutropenic, which could explain the difference between previously found increased IL-17 in IPA and the undetectable levels of IL-17 that we found. The discrepancy in data means that extrapolation of the findings obtained in the present study in rats with IPA, to patients with IPA should be done with caution. Furthermore, the use of only one strain of *A. fumigatus*, albeit from a human case of IPA, does not allow generalization of the findings in our animal model. Finally, stress in the rats during blood withdrawals could have influenced the cytokine response, however since no immune modulating anesthetics, such as isoflurane, were used, we believe the influence is limited (26-32).

From previous studies it is known that *A. fumigatus* has immunomodulatory effects. Certain constituents of the fungal cell wall are known to act as pathogen-associated molecular patterns (PAMPs), which are recognized by certain pattern recognition receptors (PRRs). β-glucan for example is recognized by C-type lectin receptor Dectin-1 and by Toll-like receptor-2 (TLR-2) and TLR-4 (33-40). Recognition of β-glucan leads to activation of multiple intracellular pathways, leading to induction of many cytokines and chemokines including GM-CSF, G-CSF, TNF-α, IL-1α, IL-1β, IL-2, IL6, IL-10, MCP-1, MIP-1α, MIP-2, IL-8 and IL-23 (33, 36, 41, 42). Tomee *et al* showed that in human epithelial cell lines exposed to *A. fumigatus* indeed IL-6, IL-8 and MCP-1 were increased (42). Although the exact receptor for chitin is not known yet, it is considered a PAMP and da Silva *et al* showed that chitin is a ligand for Dectin-1 and TLR-2 (33, 43). Stimulation with chitin

results in induction of cytokines including IL-17, IL-12/23p40, IL-23, TNF-α, RANTES and MIP-2, eventually leading to IFN-γ production (44-47). However, Chai *et al* showed that chitin has no effects on the TLR-2 and TLR-4 induced response in the host (34). Shibata showed that the size of chitin particles is important for the mounted immune response (48), which could explain these differences. Particles of 1-10 μm induce IL-12, TNF-α and IFN-γ, whereas larger particles of 50-100 μm do not induce IL-12 and IFN-γ (48). The exact size of chitin polymers or particles used in our study is not known. Since in our rat model used *A. fumigatus* conidia were used to initiate the infection, developing into hyphae and not a certain size of chitin particles, the immune response reported was induced by the combination of chitin, β-glucan and mannan. This could explain why our results do not completely match the hypothesized cytokine response for chitin particles. Based on our findings we cannot conclude whether the change in cytokines attributable to treatment with caspofungin is the result of a decrease in β-glucan exposure, or that it is caused by an increase in chitin and, by inference, leads to the activation of a different pathway.

The immunomodulatory effects of *A. fumigatus* have also been investigated. Chai *et al* showed that β-glucan modulates the host immune response through the TLR-4 pathway. β-glucan had a dose-dependent TLR-4 suppressive effect, resulting in a lower level of the pro-inflammatory IL-6 (34). Based on these findings, one would expect that a decrease in β-glucan in caspofungin-exposed *A. fumigatus* would result in higher IL-6 levels. In contrast, we found that in our caspofungin-treated rats, where the mycelium contained less β-glucan than in untreated rats, IL-6 levels were lower instead of higher. This might be explained by the mode of action of caspofungin. Treatment with caspofungin causes unmasking of galactomannan in the fungal cell wall, resulting in increased exposure of β-glucan and chitin to cell wall degrading enzymes such as chitinases and to other immune cells (39, 40, 49-52). This increased exposure is thought to activate the immune system and, thereby, contribute to the favorable clinical outcome upon administration of caspofungin (49, 50). Increased exposure of deeper cell wall layers lead to enhanced degradation of chitin into smaller particles. These smaller chitin particles skew the immune response towards Th1 instead of the Th2 response that is mounted by large chitin polymers (47). Strong *et al* confirmed that application of chitin microparticles (mostly <1 μm) increased levels of Th1 cytokines IL-12, IFN-γ and TNF-α, finally resulting in a decrease of lung inflammation (53). Again our results in the present study do not completely match this cytokine profile, probably due to the difference in immune response to chitin particles and to live *A. fumigatus*.

In summary, in the present study we demonstrated that in neutropenic rats, IPA caused a significant increase of IL-1α, IL-6, IP-10 KC, MIP-1α and MCP-1 in the lung, and of MCP-1

in serum (Figure 6). Treatment with caspofungin increases the exposure of the chitin moiety of the fungal cell wall, resulting in attenuation of the hosts' cytokine responses and in host survival.

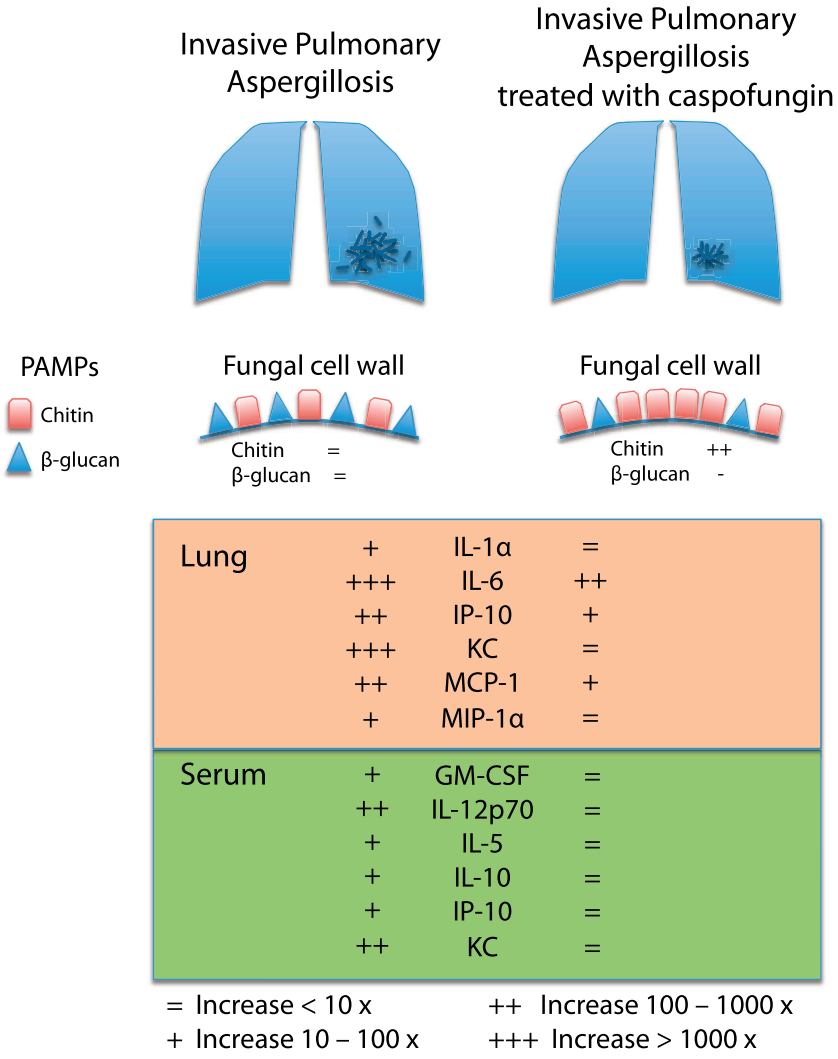

Figure 6. Summary of changes in cytokine levels in rats with IPA on day 6 after infection. The effects of treatment with caspofungin are also listed. Only cytokines that were significantly different are shown. Only cytokines that increased >10× compared to sham-infected rats were included.

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Table S1. Cytokines and chemokines that were significantly increased or decreased in lungs from infected rats with IPA compared with sham-infected rats, at various time points after infection. Only p-values that were significant (<0.05) are shown.

	Elevated or decreased in infected untreated rats			Elevated or decreased in infected, caspofungin-treated rats	
Cytokine/chemokine	Day 1	Day 3	Day 6	Day 3	Day 6
IFN-gamma	0.047				
IL-1alpha			0.016		
IL-6			0.016	0.008	0.047
IL-18	0.032			0.008	
IL-21	0.047				
IL-22		0.047			
TNF-alpha	0.032	0.032	0.016		
IP-10		0.008	0.016	0.008	
KC		0.008	0.016	0.008	0.032
MCP-1	0.029	0.008	0.016	0.008	
MIP-1alpha			0.032		0.047
RANTES	0.032			0.008	

Table S2. Cytokines and chemokines that were significantly increased or decreased in serum from infected rats with IPA compared with sham-infected rats, at various time points after infection. Only p-values that were significant (<0.05) are shown.

	Elevated or decreased in infected rats left untreated			Elevated or decreased in infected rats, treated with caspofungin	
Cytokine/chemokine	Day 1	Day 3	Day 6	Day 3	Day 6
GM-CSF	0.016				
IL-12p70	0.047				
IL-18	0.047				
TSLP	0.047				
IL-5	0.047				
IL-10	0.047				
IP-10	0.032				
KC	0.047				
MCP-1	0.032		0.016		
RANTES	0.032				
VEGF	0.047				

CHAPTER 4

Evidence supporting a role for mammalian chitinases in efficacy of caspofungin against experimental aspergillosis in immunocompromised rats

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Plos One 2013;8(10):e75844

ABSTRACT

Caspofungin, currently used as salvage therapy for invasive pulmonary aspergillosis (IPA), strangely only causes morphological changes in fungal growth *in vitro* but does not inhibit the growth. *In vivo* it has good efficacy. Therefore the question arises how this *in vivo* activity is reached. Caspofungin is known to increase the amount of chitin in the fungal cell wall. Mammals produce two chitinases, chitotriosidase and AMCase, which can hydrolyse chitin. We hypothesized that the mammalian chitinases play a role in the *in vivo* efficacy of caspofungin.

In order to determine the role of chitotriosidase and AMCase in IPA, both chitinases were measured in rats which did or did not receive caspofungin treatment. In order to understand the role of each chitinase in the breakdown of the caspofungin-exposed cells, we also exposed caspofungin treated fungi to recombinant enzymes *in vitro*.

IPA in immunocompromised rats caused a dramatic increase in chitinase activity. This increase in chitinase activity was still noted when rats were treated with caspofungin. *In vitro*, it was demonstrated that the action of both chitinases were needed to lyse the fungal cell wall upon caspofungin exposure.

Caspofungin seemed to alter the cell wall in such a way that the two chitinases, when combined, could lyse the fungal cell wall and assisted in clearing the fungal pathogen. We also found that both chitinases combined had a direct effect on the fungus *in vitro*.

INTRODUCTION

Aspergillus fumigatus is a ubiquitous saprophytic fungus, producing conidia that are inhaled daily by humans. Usually inhalation of these spores causes no problem; however, *A. fumigatus* can cause a broad range of diseases in hosts with underlying conditions. Patients with inflammatory conditions, such as asthma and cystic fibrosis, can develop allergic bronchopulmonary aspergillosis (ABPA)(1, 2). Patients with prolonged neutropenia are at risk to develop invasive pulmonary aspergillosis (IPA)(3). In neutropenic patients, IPA may be characterized by necrotizing pneumonia or hemorrhagic infarctions. Due to the progressive character of IPA, the morbidity and mortality is high. The response to treatment is limited, despite the application of several antifungal agents with different mechanisms of action.

The current first choice antifungal agent for IPA is voriconazole, with amphotericin B as the alternative therapy (3). Caspofungin is available as salvage therapy, in case of refractory disease or intolerance of voriconazole or amphotericin B by the patient. Strikingly, *in vitro* caspofungin has only a mediocre activity against *A. fumigatus*. It does not have fungicidal or fungistatic activity. Only at a very high concentration growth inhibition is noted. However, in *in vivo* animal models caspofungin seems to be very potent. In our transiently neutropenic rat model with unilateral invasive pulmonary aspergillosis, the human equivalent dosage of caspofungin results in 100% efficacy (4). Therefore the question arises what the reason is for this discrepancy between *in vivo* and *in vitro* results.

One hypothesis would be that the immune system of the host plays a role. Caspofungin acts by inhibiting β-glucan synthase thereby decreasing the β-glucan contents of the fungal cell wall. To restore the stability of the cell wall, the fungus reacts by increasing its other major cell wall component, chitin, as demonstrated *in vitro* (5, 6).

Chitin can be cleaved by chitinases, which belong to family 18 of glycosyl hydrolases and are produced in cooperation with the immune system. Chitinases are classified into endochitinases and exochitinases. Exochitinases act at the non-reducing ends of chitin with the release of successive diacetyl chitobiose units. In contrast, endochitinases randomly cleave at internal points in the chitin chain (7, 8). Mammals are known to produce two types of chitinases: chitotriosidase and acidic mammalian chitinase (AMCase)(9). Chitotriosidase is produced by macrophages and polymorphonuclear neutrophils (10, 11) and can be found in the lungs of mammals (12, 13) as well as in lacrimal glands (14). AMCase is an exochitinase produced by macrophages and epithelial cells (15) and is found mainly in the gastro-intestinal tract of mammals to digest nutritional chitin, though it was also found in the lung at low concentrations (10). The exact role of chitin-

ases remains to be clarified. However, an important role for chitinases in allergic diseases has been suggested (16, 17).

In the past it was already demonstrated by Overdijk *et al* that chitinase activity was increased in plasma isolated from guinea pigs with a systemic *A. fumigatus* infection. Furthermore, high chitinase levels were detected in the spleen, followed by lungs and kidneys (18, 19). However, these results did not reveal whether the chitinase activity was due to chitotriosidase or AMCase activity, or a combination of both since at that time, AMCase had not been discovered as a chitinase yet (10).

Summarizing the above, we can state that the chitin content in the *A. fumigatus* cell wall is increased upon exposure to caspofungin and mammalian chitinases are induced during invasive aspergillosis. We therefore hypothesized that either chitotriosidase, AMCase or both play a role in the clearing of *A. fumigatus* from the lung when treated with caspofungin. In order to test this hypothesis, we first determined which of these two chitinases was induced upon *A. fumigatus* conidia in both immunocompetent rats, clearing the conidia, and in immunocompromised rats, suffering from invasive pulmonary aspergillosis. In these experiments we determined if chitinases could be induced in the first place during the neutropenic state. Next, we determined if both chitotriosidase and AMCase were more extensively expressed during caspofungin treatment. Afterwards we investigated how these two chitinases and caspofungin interact and what the combined effect is on the *A. fumigatus* hyphae *in vitro*.

MATERIALS & METHODS

Experimental animal model

The rat model of invasive pulmonary aspergillosis (IPA) in immunocompromised rats used, was described previously (20). Some minor changes have led to the following experimental set up.

In order to determine if *Aspergillus fumigatus* conidia induce chitinase activity, immunocompetent female albino RP rats were inoculated intratracheally with a clinical isolate of *A. fumigatus* originally isolated from a hemato-oncological patient with IPA. Left-sided pulmonary inoculation was established by intubation of the left main bronchus, while the rats were under general anaesthesia. A cannula was passed through the tube and the left lung was inoculated with 20 µl phosphate buffered saline (pbs) containing 6×10^4 conidia of *A. fumigatus*. Rats were sacrificed at day 1, 3 and 6 after fungal inoculation to determine the chitotriosidase and AMCase activity both enzymatically and immunohistochemically. For this, blood samples were taken by puncture of the orbital plexus and rats were sacrificed by CO_2 exposure. The left lung was removed and either stored at

−80°C until analysis, or fixated in formalin for immunohistochemistry. Serum was also stored at −80°C until analysis. The groups consisted of a minimum of 4 rats. Infected organs and blood from rats found dead were always cultured to exclude bacterial superinfections. Fungal load was assessed by determination of serum galactomannan index (GM-index), using the commercially available Platelia *Aspergillus* EIA Platelia *Aspergillus* system of BioRad (Marnes-la-Coquette, France).

In order to determine if *A. fumigatus* conidia induce chitinase activity in neutropenic female albino RP rats developing IPA, transient neutropenia was induced by intraperitoneally (i.p.) administered cyclophosphamide (Endoxan, Baxter, Utrecht, The Netherlands) in doses of 75, 60, 50 and 40 mg/kg bodyweight at 5 and 1 days before fungal inoculation, and at 3 and 7 days after fungal inoculation, respectively. Whereas the normal leukocyte counts in our rats is 5.8×10^9/L, the leukocyte counts decreased following cyclophosphamide treatment and were 6.5×10^7/L on the day of fungal inoculation and 6.4×10^7/L on day 5 and day 9 after inoculation. After the last dosage of cyclophosphamide, leukocyte counts rose to 2.6×10^9/L on day 13 and to 6.0×10^9/L on day 21 after infection. Granulocyte counts decreased from 2×10^8/L before cyclophosphamide was given (normal counts in our rats) to 2×10^4/L from the day of fungal inoculation to day 9. After the final dosage of cyclophosphamide, granulocyte counts increased again to 1.2×10^7/L and 1.2×10^9/L on day 13 and 21 after infection, respectively.

To prevent bacterial superinfections, rats were given ciprofloxacin (500 mg/L) and colistin (100 mg/L) in their drinking water. Furthermore, rats were given teicoplanin intramuscularly (i.m.) in doses of 30 mg/kg on days 5 and 1 pre- inoculation, and 15 mg/kg on days 1, 3, 6, 8 and 10 post-inoculation. Immunocompetent rats were injected with saline i.p. instead of cyclophosphamide.

Left-sided pulmonary infection was established, by intubation as described for the immunocompetent rats. Again, rats were sacrificed at day 1, 3 and 6 after fungal inoculation to determine the chitotriosidase and AMCase activity both enzymatically as immunohistochemically as described for the immunocompetent rats.

Since rats were sacrificed at these predetermined time points, death of rats was no primary endpoint. Rats were monitored according to a discomfort scale by the researchers several times a day, during the entire experiment. In order to limit suffering, rats were euthanized in case of high discomfort, shown by e.g. increased breathing exercise, increased respiratory rates and altered behaviour, like decreased movements and unkempt appearance (dull haircoat). The experimental protocols adhered to the rules specified in the Dutch Animal Experimentation Act (1977) and the *Guidelines on the Protection of Experimental Animals* published by the Council of the EC (7a). The present protocols were approved by the Institutional Animal Care and Use Committee of the Erasmus MC Rotterdam.

Antifungal treatment

Caspofungin (Merck & Company, Rahway, NJ, USA) was diluted in saline and administered intraperitoneally once daily in a dose of 4 mg/kg/day. Treatment was started at 24h (early stage IPA) or at 72h (late stage IPA) after fungal inoculation. Treatment was continued for six days.

Recombinant expression of rat AMCase

Recombinant rat AMCase was generated using the pMIB insect cell expression system by Invitrogen as previously described for human chitotriosidase (14). The primers had *SphI* (forward primer) and *Xba*1 (reverse primer) restriction sites at the 5′ ends that facilitated the in-frame cloning into pMIB/V5-His (Invitrogen). The primer sequences were as follows: RnCHIA-F 5′-GCCCGGGCATGCATtacaatctggtatgctacttcac-3′ and RnCHIA-R 5′- GCCCGGTCTAGAtggccagttgcagcaattacagctg-3′ (restriction sites underlined). The PCR reactions for generation of full length AMCase for expression cloning were conducted using *Pfu* DNA polymerase (Stratagene) following the manufacturer's instructions for a 50µl reaction using cDNA obtained from rat lung tissue. The final sequence of the recombinant plasmid was confirmed by DNA sequencing. The activity of the recombinant rat AMCase produced here and the purchased recombinant chitotriosidase were determined with the chitinase assays as described below.

Chitinase assay

Chitinase activity was determined in homogenized lung tissue. Activity of 4-Methylumbelliferyl N,N′-diacetyl-β-D-chitobioside (chitobiosidase activity, corresponding with AMCase activity) and 4-Methylumbelliferyl β-D-N,N′,N″-triacetylchitotriose (endochitinase activity, corresponding with chitotriosidase activity) were determined using the commercial fluorimetric Chitinase Assay Kit (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Chitinase activity in each sample was measured in duplicate. Chitinase activity was expressed in arbitrary units (a.u.) and median chitinase activity levels were compared for different groups of rats. Chitinase activity from *Trichoderma viride* (control enzyme from the Chitinase Assay Kit described above) was used as a positive control. A positive and a negative control were used in each run, in order to validate the experiment.

Chitotriosidase and AMCase activity were measured in homogenized lungs. It was confirmed that all chitinase activity was indeed of host origin as follows: *A. fumigatus* conidia were cultured for 48 hours at 37°C in Sabouraud's broth or RPMI, with and without caspofungin 1 mg/L. The broth was filtered after 48h and chitinase activity in the broth was measured in triplicate. Activity of chitotriosidase and AMCase was measured with the commercial fluorimetric Chitinase Assay Kit and was found to be < 5 a.u. in all

samples tested. We thus concluded that the chitinases measured in the lung tissue were indeed of host origin and not of fungal origin.

Immunohistochemistry of lungs and *in vitro* cultures

Lungs were fixed in formalin, embedded in paraffin and processed for immunohistochemical evaluation. First, lungs were deparaffinised in xylene, then rehydrated in decreasing concentrations of ethanol. Endogenous peroxidase was blocked in methanol with 0.3% H_2O_2 and non-specific binding sites were blocked with rabbit or goat serum. Subsequently, coupes were incubated overnight with rabbit polyclonal antibody directed against chitotriosidase (H-66, 1:75, Santa Cruz Biotechnology, Santa Cruz, USA) or with goat polyclonal antibody directed against AMCase (Y-14, 1:50, both Santa Cruz Biotechnology, Santa Cruz, USA). As a control we used a goat polyclonal IgG antibody directed against swine IgM (A100-100A, 1:50, Bethyl Laboratories, Montgomery, USA). From the VectaStain® Elite ABC kit (Vector Laboratories Burlingame, CA, USA), anti-rabbit IgG or anti-goat IgG was used as a secondary antibody and the coupes were developed using the protocol from the kit. Hematoxylin was used as counter staining. In order to ascertain that the antibodies did not react with chitinases expressed by *A. fumigatus* itself, *A. fumigatus* was grown on the same histological slides and fixated. These fixated *A. fumigatus* slides were stained according to the same protocol used for the histological slides. No staining of either chitotriosidase or AMCase was observed.

In vitro binding of chitinases to fungal hyphae

Aspergillus fumigatus was cultured for 48h at 37°C on Sabouraud's agar with or without 1 µg/ml caspofungin, on which a cover slip had been placed. The fungus adhered to the cover slip and after 48h the cover slips were removed and processed. Cover slips were incubated with 0.5 mg/L recombinant chitotriosidase (purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany) or with 0.5 mg/L recombinant AMCase (recAMCase; School of Pharmacy, Nottingham, UK), both or with aquadest for 2 hours at 37°C. Afterwards, cover slips were fixed in 70% and 100% ethanol. Non-specific binding sites were blocked with rabbit or goat serum. Cover slips were further processed identical to the lung coupes; however no counter staining was used.

Determination of *in vitro* inhibitory concentrations

Minimal inhibitory concentrations (MICs) were determined for recAMCase, recChito and chitinase from *T. viride* (see section "Chitinase assay") according to the microdilution methods described by the CLSI (21). Final concentrations of recAMCase were 0.031 – 128 mg/L. Final concentrations of recChito were 0.031 – 16 mg/L. Final concentrations of chitinase from *T. viride* were 0.031 – 16 mg/L. Minimal effective concentration (MEC) was

determined for caspofungin, according to the same guidelines (21). Final concentrations of caspofungin were 0.063 – 128 mg/L.

Checkerboard titrations were conducted in triplicate for caspofungin combined with recAMCase, recChito and chitinase from *T. viride* as a positive control and fractional inhibitory concentration indices (FICIs) were calculated as previously published (6). MEC was used for caspofungin and MICs were used for chitotriosidase and AMCase. Drug interactions were classified as synergistic (FICI ≤ 0.5), indifferent (0.5 < FICI < 4) or antagonistic (FICI ≥ 4).

Fungal cell wall assessment by fluorescent microscopy

Aspergillus fumigatus was cultured on a cover slip as described above. Cover slips were incubated with either recombinant chitotriosidase alone, recombinant AMCase alone, both recombinant chitinases or with aquadest as negative control for 2 hours at 37°C. Cover slips were washed in aquadest and incubated with 25 µM Calcofluor White (Molecular Probes®, Leiden, The Netherlands) for 30 minutes at 37°C in the dark. Afterwards, cover slips were washed in aquadest and placed on a microscopic slide and assessed by fluorescent microscopy.

Statistics

Differences in chitinase activity levels between groups were analysed using the Mann-Whitney U-test (GraphPad Prism Software, San Diego, USA). A p-value of < 0.05 was considered significant.

RESULTS

Chitinases in immunocompetent rats inoculated with *A. fumigatus*

In order to determine if *A. fumigatus* conidia induce chitinase activity, immunocompetent rats were inoculated intratracheally with *A. fumigatus* conidia. After inhalation of the inoculum, all rats remained asymptomatic and none of the rats developed invasive pulmonary aspergillosis (IPA), their serum galactomannan indices remained <0.2 and no hyphae were observed in Grocott-stained lungs on day 1, day 3 and day 6. Compared to the situation before inoculation, both chitotriosidase and AMCase activity in the lung increased on day 1 after inoculation, from 25 to 119 arbitrary units (a.u.)(chitotriosidase; p=0.011) and from 17 to 60 a.u. (AMCase; p=0.006). After the initial increase in chitinase activity on day 1, enzyme activity of both chitinases remained elevated on day 3 and day 6 but did not increase further when compared to day 1 (p>0.05; data not shown). Thus, exposure of the rats to conidia resulted in increased activity of both chitotriosidase and AMCase for several days. However, the data obtained with immunohistochemistry

suggested that inoculation with conidia did not result in increased presence of chitotriosidase and AMCase in the lung.

Chitinases in immunocompromised rats with IPA

To determine if chitinases were also induced during the neutropenic state, we investigated chitinase activity in immunocompromised rats. Neutrophil depletion was induced using cyclophosphamide as described in the experimental procedures section and immunocompromised rats were inoculated intratracheally with *A. fumigatus* conidia. As previously described, all rats developed IPA and died within ten days, if left untreated (20). Bacterial superinfections were never found. IPA was confirmed by galactomannan

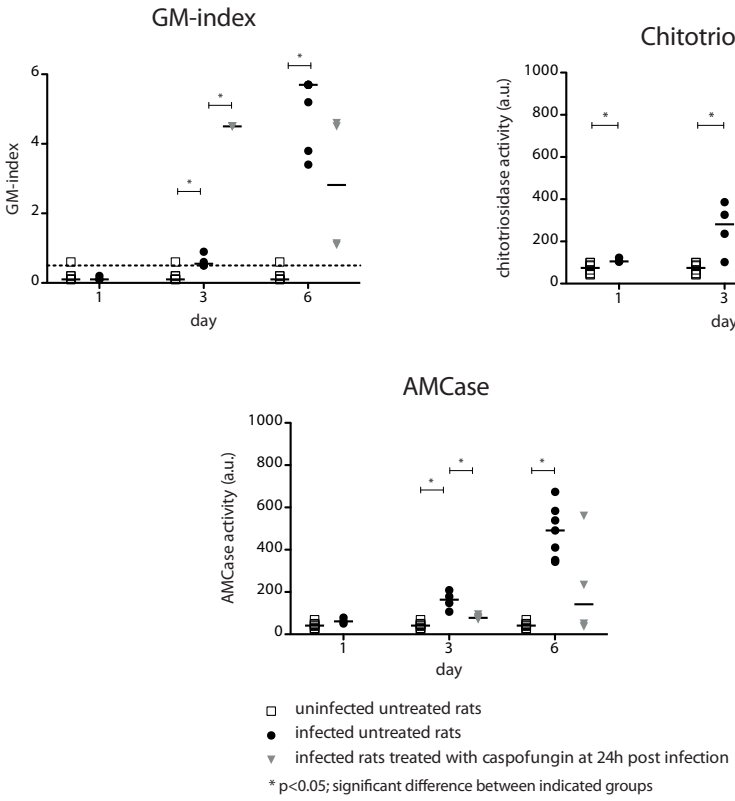

Figure 1. Galactomannan (GM) index and chitinase activity in immunocompromised rats inoculated with *A. fumigatus* conidia. Squares: uninfected untreated rats; circles: infected untreated rats; triangles: infected rats, treated with caspofungin at 24h post infection. Bars represent medians. For each group n ≥ 4.
A. GM-index, measured by Platelia assay. GM-index of <0,5 is considered negative.
B and C. Chitotriosidase and AMCase activity, expressed in arbitrary units (a.u.). *p<0.05; significant difference between the indicated groups.

indices in serum (Figure 1A) and histopathology of the lung (Grocott staining; Figure 2D), which showed invasive fungal disease.

Chitotriosidase and AMCase activity increased during fungal infection (Figure 1B, C).

Chitotriosidase activity increased from 75 a.u. before inoculation to 105 a.u. on day 1 ($p=0.041$), to 281 a.u. on day 3 and to 501 a.u. on day 6 (both $p<0.001$). AMCase activity increased from 41 a.u. before inoculation to 61 a.u. on day 1 ($p=0.066$), 164 a.u. on day 3 and 491 a.u. on day 6 (both $p< 0.001$; see Figure 1B,C). The initial increase in chitinase activity on day 1 was similar to that in immunocompetent rats. However, immunocompromised rats developed invasive fungal disease and the chitinase activities further increased, indicating that a higher fungal content was related to both a higher chitotriosidase and AMCase activity in the lung. Both chitotriosidase and AMCase activity were host specific, since no activity was found when *A. fumigatus* culture supernatant were measured.

The histology of lungs of immunocompromised rats with IPA showed disturbed lung morphology. There was an inflammatory area around fungal foci, where the normal structure of alveoli was lost (Figure 2D, E, F). After inoculation, AMCase and chitotriosidase were increasingly expressed around the fungal focus (Figure 2E, F), which was consistent with the increased chitinase activity levels as described above. The staining of both chitinases was deemed specific for host chitinases, since no staining was observed when *A. fumigatus* was cultured on a slide and stained afterwards (see above).

Chitinase activity in immunocompromised rats with IPA treated with caspofungin

In order to determine if chitinase expression and activity differed in rats treated with caspofungin, another group of immunocompromised rats was inoculated with *A. fumigatus*. Treatment with caspofungin was started at 24h post fungal inoculation, representing early stage IPA. Treatment started at this early time point resulted in survival of 90% of the rats (20). Chitotriosidase and AMCase activity initially increased after fungal inoculation, as described above. In infected caspofungin-treated rats, at day 3 chitotriosidase and AMCase levels were similar to the levels in infected untreated rats (Figure 1B, C). On day 6, a trend was observed that chitotriosidase and AMCase levels were lower in infected caspofungin-treated rats compared to infected untreated rats (Figure 1B, C), however the differences were not statistically significant. The same trend was found for fungal load, in terms of GM-index (Figure 1A); GM-index was 5.7 for untreated rats and 2.8 for treated rats ($p=0.053$). On day 3, GM-index was lower for untreated rats than for caspofungin-treated rats, in contrast to chitinase activity. Apparently, chitinase activity is increased before GM-index rises.

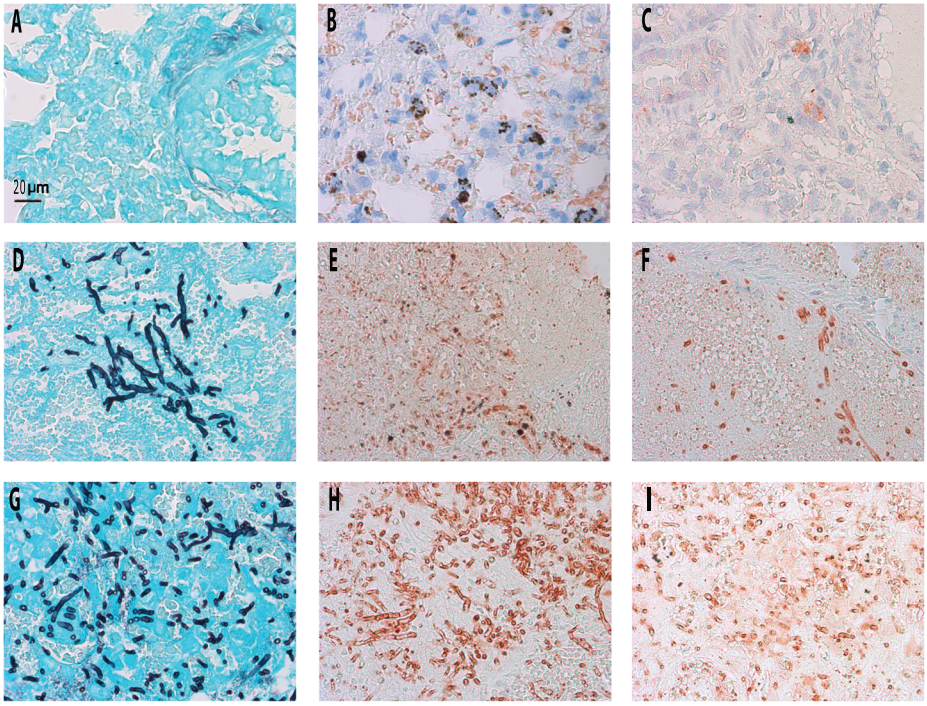

Figure 2. Grocott staining (A, D, G) and presence of AMCase (B, E, H) and chitotriosidase (C, F, I) in several rats. Panels A, B, and C show the lung of an uninfected rat. Panels D, E and F show the fungal focus in an infected, untreated rat. Panels G, H and I show the fungal focus in an infected, caspofungin treated rat. Original magnification × 400. Scale bar 20 µm. All panels represent lungs on day 6 after inoculation. Slides were stained according to the described protocols. In Grocott staining (A, D, G), fungal hyphae are coloured black. Chitotriosidase- or AMCase-presenting cells are coloured red (B, C, E, F, H, I).
In uninfected rats, normal morphology can be found in the lungs (A, B, C). In infected rats, normal morphology of alveoli is lost (D, E, F). Grocott staining shows many hyphae (D). An inflammatory response is found around the fungal focus, where chitotriosidase and AMCase are increasingly present (red zones) compared to an uninfected rat (E, F). After treatment with caspofungin, Grocott staining shows fungal material in an infected rat (G). AMCase bound fungal hyphae after treatment with caspofungin (H) and thus hyphae became visible. After treatment with caspofungin, chitotriosidase seemed to also bind the fungal cell wall and locate inside hyphal cells (I).

Immunohistochemistry showed a similar pattern of expression of chitotriosidase and AMCase in infected caspofungin-treated and infected untreated rats. Expression of both chitinases was highest around the fungal focus. In other relatively healthy parts of the lung, expression of chitinases was similar to that in uninfected rats. Figure 2 shows the fungal focus and the location of the AMCase and chitotriosidase expressing cells. AMCase was expressed by several cells throughout the lung, though expression was also highest around the fungal focus (Figure 2E). After treatment with caspofungin, AMCase was found to bind to the fungal cell wall (Figure 2H), though not all hyphae

were stained. This was not seen in hyphae of untreated animals. In unaffected parts of the infected lung, where normal morphology was maintained, AMCase expression was comparable with that in uninfected rats (data not shown). Without caspofungin treatment, chitotriosidase was found also mainly around foci of fungal growth. No distinct cell type expressing chitotriosidase could be assigned (Figure 2F). After treatment with caspofungin, chitotriosidase was found to bind the hyphal cell wall and inside the cell (Figure 2I). Expression of both chitinases was highest in close proximity to the fungal focus, regardless of treatment. Apparently, treatment with caspofungin caused such an alteration in the fungal cell wall that AMCase was able to bind the fungal hyphae and chitotriosidase was taken up by the fungus.

In order to investigate late stage IPA, we also determined chitinase activity levels in a group of rats that received caspofungin treatment starting at 72h post fungal inoculation (late stage IPA). GM-index and immunohistochemistry revealed increased fungal load compared to rats that were treated with caspofungin starting at 24h post inoculation (early stage IPA). For chitinase activity, we observed levels that were not significantly different between the two groups (data not shown).

In vitro binding of recombinant chitinase to hyphae of *A. fumigatus*

As described above, we observed binding of AMCase and hyphal uptake of chitotriosidase in the lung after treatment with caspofungin. We hypothesized that treatment with caspofungin was required for binding of both chitinases, since caspofungin causes increased chitin contents in the fungal cell wall (6) and thus increases the substrate for chitinases. In order to confirm our hypothesis, we investigated *in vitro* binding of recombinant AMCase and recombinant chitotriosidase to *A. fumigatus* hyphae on slides. *A. fumigatus* was cultured on cover slips in presence or absence of caspofungin, which was followed by exposure to recombinant AMCase or recombinant chitotriosidase or both for two hours. Binding of the enzyme was detected in the same way as expression was detected in the lungs taken from rats with IPA. Additionally, we tested antifungal activity of recombinant AMCase or recombinant chitotriosidase alone and combined with caspofungin in a checkerboard titration according to the CLSI criteria.

Recombinant AMCase did bind similarly to fungal hyphae both in unexposed and in caspofungin-exposed fungal cells (Figure 3C, F) showing that caspofungin exposure was not needed for binding of AMCase. Susceptibility testing showed a median minimal inhibitory concentration (MIC) of recombinant AMCase of >16 mg/L. Combination of recombinant AMCase with caspofungin in a checkerboard titration showed no synergy (median fractional inhibitory concentration index (2) 2.0). Thus exposure to caspofungin and recombinant AMCase was not sufficient for antifungal activity *in vitro*.

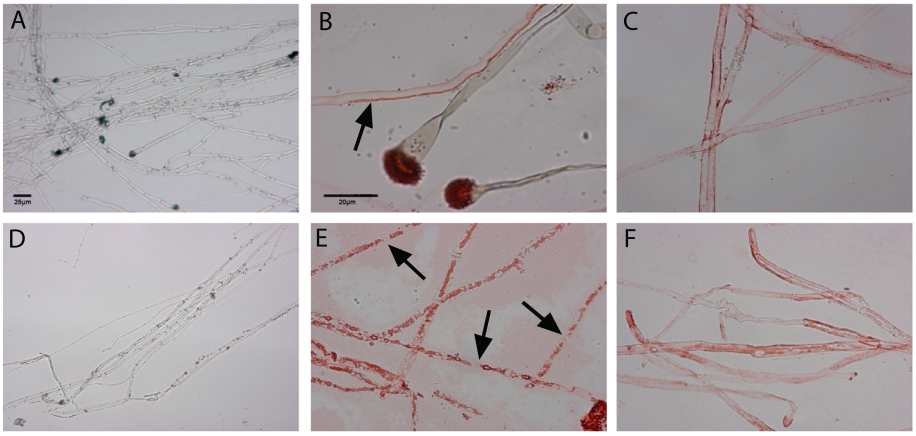

Figure 3. *In vitro* binding of recombinant chitinases to *A. fumigatus* hyphae. Binding of recombinant chitotriosidase (A, D). Binding of recombinant chitotriosidase when incubated in combination with recombinant AMCase (B, E) and binding of recombinant AMCase (C, F). Panels A, B and C show unexposed hyphae. Panels D, E and F show caspofungin-exposed hyphae. A, D Original magnification ×100, scale bar 25 µm. B, C, E, F Original magnification ×400, scale bar 20 µm. Slides were stained according to the described protocols. Binding of either recombinant enzyme is characterized by a red colour. A, D Recombinant chitotriosidase did not bind to unexposed hyphae (A) or to caspofungin-exposed hyphae (D). B, E When incubated with a combination of recombinant chitotriosidase and recombinant AMCase, recombinant chitotriosidase did bind to unexposed hyphae (arrow) and to conidial heads (C) and seemed to be taken up by the fungal cells after caspofungin exposure (E). Also the cell wall seemed to be lysed at several locations (arrows). C,F Recombinant AMCase did bind to unexposed (C) and to caspofungin-exposed hyphae (F).

Recombinant chitotriosidase was not taken up by *A. fumigatus* hyphae during incubation with this enzyme regardless of caspofungin exposure (Figure 3A, D), in contrast to what was seen in infected lungs. Susceptibility testing showed an MIC of recombinant chitotriosidase of >16 mg/L. Combination of recombinant chitotriosidase with caspofungin in the checkerboard titration showed again no synergy (median FICI 2.0). Thus exposure to caspofungin and recombinant chitotriosidase alone was also not sufficient for antifungal activity *in vitro*. Strikingly the binding pattern of recombinant chitotriosidase in the infected lungs was different to the pattern found *in vitro*. *In vivo*, both chitinases were present, though only one chitinase was stained at a time. Hence we additionally investigated the binding of recombinant chitotriosidase to the fungus in presence of both recombinant enzymes *in vitro*. When hyphae were not exposed to caspofungin, chitotriosidase seemed to locate inside the hyphae and was also bound to the conidial heads (Figure 3B). When hyphae were exposed to caspofungin and to both recombinant chitinases, chitotriosidase also located inside the cell wall and strikingly, the cell wall seemed to dissolve (Figure 3E). From these experiments, we observed that recombinant chitotriosidase could only be taken up by hyphae, provided recombinant AMCase was present. Furthermore we observed that caspofungin modifies the fungal

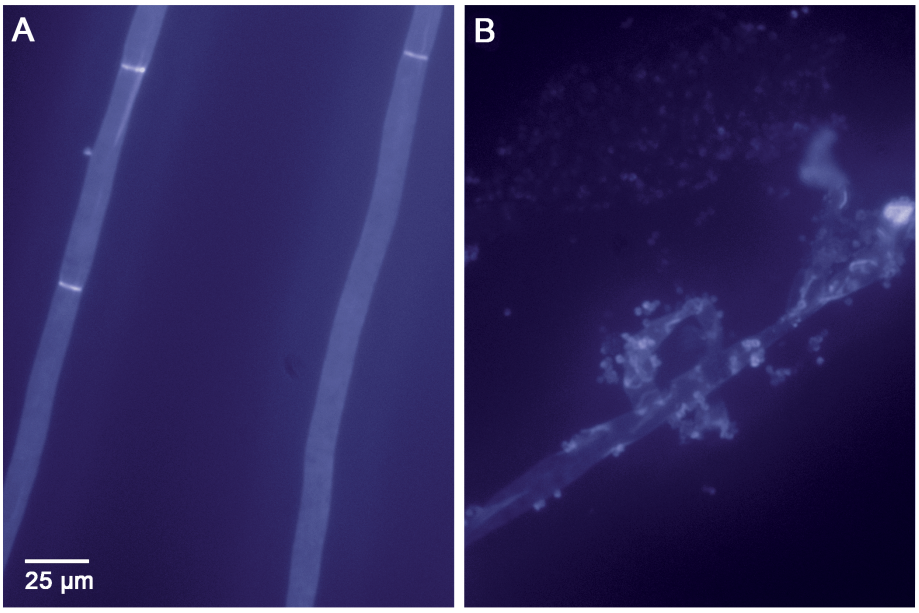

Figure 4. Calcofluor White staining of *in vitro* cultured *A. fumigatus* hyphae after incubation with recombinant chitotriosidase and recombinant AMCase. Magnification ×400, scale bar 25 µm. When hyphae were cultured on Sabauroud's agar (A), the cell wall remained regular and intact after incubation with the two recombinant chitinases. When hyphae were cultured on Sabauroud's agar with 1 mg/L caspofungin (B), the cell wall was irregular and disrupted after incubation with the two recombinant chitinases.

cell wall in such a way, that a combination of recombinant AMCase and recombinant chitotriosidase can lyse the fungal cell wall. The uptake of chitotriosidase and morphologic disruptions suggest chitinolytic results and thus clinical significance in the clearance of fungal material.

We aimed to confirm this important finding in an alternative experiment. It was not possible to conduct a checkerboard titration with caspofungin and both recombinant chitinases, due to limited availability of recombinant enzymes. Other types of viability assays were unfortunately also not successful. Instead of a viability assay, we then stained the fungal cell wall with Calcofluor White after exposure to caspofungin and both recombinant chitinases. Figure 4A shows unexposed fungal cells and Figure 4B shows caspofungin-exposed cells, both after incubation with a combination of recombinant chitotriosidase and recombinant AMCase. These panels are representative for the complete culture. In other words, most fungal hyphae that were exposed to both chitinases and caspofungin were disrupted. The cells unexposed to caspofungin look very regular with a normal cell wall, with Calcofluor staining mainly seen in regularly spaced septa, whereas the caspofungin-exposed cells show an irregular cell wall with a fragmented aspect. When fungal cells were incubated with a single recombinant chitinase, the cell

wall remained regular. When fungal cells were exposed to caspofungin only, the cell wall looked very regular and similar to that in Figure 4A. Thus, in this assay, we confirmed the earlier observations of disruption of the fungal cell wall after exposure to caspofungin and both chitinases. The observed damage to the fungal cell wall makes it very unlikely that the fungal cells remain viable.

DISCUSSION

The role of mammalian chitinases in the response of invasive fungal infections is a relatively unexplored area of research. In the past it has been shown that chitinase activity was increased in guinea pigs upon *A. fumigatus* systemic infection, but it was not known which chitinase was involved (18, 19). In the present study we confirmed that chitinases in the lung of immunocompetent rats and of immunocompromised rats were increased after exposure to *A. fumigatus* conidia. We demonstrated that both chitotriosidase and AMCase play a role. We showed that in immunocompetent rats with an appropriate immune system, transient exposure to a high load of conidia without subsequent lung infection resulted in only a moderate increase in the production and activity of chitinases in the lung. Based on our findings, we expect that in certain groups of immunocompetent *A. fumigatus*-colonized patients (such as cystic fibrosis patients), chitinase activity levels would be slightly increased on a permanent basis. We studied chitinase activity in rats with IPA, though extrapolation to the human situation should be done with care.

In all immunocompromised rats developing IPA after inhalation of conidia, chitinases were produced, even though the rats were in neutropenic state. Since *A. fumigatus* itself also produces chitinases, we had to determine if the chitinases measured with our assays were of host origin. We therefore included *in vitro* controls for both the enzyme-assays and immunohistochemistry experiments. These controls were prepared by growing *A. fumigatus in vitro*, without animal cells. No enzyme-activity was observed with the substrates used and no binding of the AMCase and chitotriosidase was observed. Since *in vitro* simulations are not completely representative for the *in vivo* situation, it is not easy to rule out that the fungal chitinases, which might be induced *in vivo* only, were not cross-reacting in our assays. The assays used in our study were similar to the assays used by Overdijk *et al* (18, 19). He demonstrated with a Bio-Gel P-100 gel filtration assay that the chitinase activity in the lung consisted of two peaks, one of 35 kDa and one of 15 kDa, which both appeared to be true chitinases (19). To rule out a fungal origin of the chitinase activity measured in the lungs of *A. fumigatus* infected guinea pigs, Overdijk *et al* demonstrated that the *A. fumigatus* chitinases eluted much earlier from the column

than the mammalian chitinases (19). Furthermore, allosamidin reduced the chitinase activity in infected and in uninfected guinea pigs by 94% (18). His final proof was that the activity ratio with the substrates 4-methylumbelliferyl-*N*-acetylglucosamine and 4-methylumbelliferyl-*N*-acetylgalactosamine differed significantly between *A. fumigatus* chitinases and chitinases present in guinea pig serum (18).

It is known that chitotriosidase and AMCase are produced by several cells, such as alveolar macrophages, epithelial cells and neutrophils (11, 22, 23). Our observations show that chitinase activity was increased significantly during *A. fumigatus* infection, even in rats with decreased numbers of neutrophils and macrophages. During progression to IPA, both the amount of fungal material and the chitinase activity increased in the lung over time.

Treatment with antifungal agents reduces the fungal mass present in the lung. Therefore it was not surprising that Overdijk *et al* found that treatment of systemic aspergillosis with itraconazole or amphotericin B limited the increase in chitinase activity in guinea pigs (18). Caspofungin belongs to another class of antifungal agents. It restricts the growth of *A. fumigatus* in the lung and it alters the cell wall composition by decreasing the amount of β-glucan and increasing the amount of chitin in the fungal cell wall (6). Since caspofungin both reduces the fungal mass in the lung but also increases the relative chitin concentration within this mass, resulting in more substrate for the chitinases, the chitinase activity could either be decreased or increased upon treatment. In our study we found a trend towards a blunting of the chitinase response following treatment with caspofungin, suggesting that the total amount of fungal cells seems to be more important than the composition of the cell wall in the induction of chitinase expression. The total chitinase activity was lower in lungs of caspofungin treated rats than in untreated rats. In contrast, expression of both chitinases seemed to be higher around the fungal focus in caspofungin treated rats. This could be explained by the methods used. Chitinase activity was measured in homogenate of a complete lung, whereas the immunohistochemistry shown displays only the fungal focus. Thus the images shown are not representative for the complete lung, since the fungal focus is located in only a limited segment of the lung. Furthermore it became clear again that caspofungin does not have fungicidal or fungistatic activity. This was supported by the high GM-index of infected, caspofungin-treated rats (Figure 1A). The high GM-index on day 3 could be explained by the detachment of the galactomannan chain from the β-glucan polymeres, caused by caspofungin treatment. Caspofungin is currently in use as salvage therapy for fungal infections. Future studies will be performed to determine if the chitinases also work synergistic with other antifungal agents such as the azoles and polyenes.

Although total chitinase activity in infected rats was not influenced by treatment with caspofungin, the location of the individual chitinases was substantially changed. Upon

caspofungin treatment, both AMCase and chitotriosidase seemed to bind to the fungal hyphae, which was not found in untreated infected rats. Furthermore, galactomannan is released from the cell wall resulting in the unmasking of β-glucan and chitin on the *A. fumigatus* cell wall. Unmasking β-glucan has been shown to result in an increased inflammatory response compared to untreated hyphae (24). The unmasking of the chitin polymers, the target of the chitinases, seems to cause enhanced binding of chitinases to chitin. Possibly, treatment with caspofungin increased the available mammalian chitinase binding domains in the fungus, which might be responsible for the good clinical outcome of treatment with this agent.

It appeared that AMCase was located on the fungal cell wall, while chitotriosidase was located inside the fungal cells. The observed expression patterns suggest that AMCase and chitotriosidase each have a distinct target location and bind to different parts of the fungal cell. It was shown that recombinant AMCase *in vitro* indeed bound the hyphae but that recombinant chitotriosidase was taken up by hyphae only after exposure to both chitinases, thus mimicking the *in vivo* situation where also both chitinases are present. It seemed that recombinant chitotriosidase needed the exochitinase activity of recombinant AMCase in order to be taken up by the fungus. This suggests a synergy between endochitinases and exochitinases. Bolar *et al* also suggested that endochitinases and exochitinases act synergistically in plants (25). They showed that plants expressing both types of chitinases were less susceptible to *Venturia inaequalis*, the fungal causal agent of apple scab, than plants expressing one of the chitinases (25). Our observations suggest that such a type of synergy may also be present in mammals.

The synergy between chitotriosidase and AMCase seemed most important when *A. fumigatus* was exposed to caspofungin *in vitro*. When *A. fumigatus* was incubated with caspofungin and both chitotriosidase and AMCase, a direct cell wall degrading effect was noted. No degradation was found when only one of the chitinases was used or when the fungus was not exposed to caspofungin. Also, when the *in vitro* susceptibility assays were performed with each chitinase alone, or when combined with caspofungin, no direct fungicidal effect was observed. Several other authors also investigated activity of chitotriosidase and AMCase, with varying results (11, 26). Differences in experimental setup could explain the differences found in antifungal activity.

The cell wall degrading properties of both chitinases in combination with exposure to caspofungin could explain why treatment with caspofungin results in decreased mortality in IPA, in spite of the limited fungicidal properties *in vitro*. However, chitotriosidase and AMCase are not expected to be the only type of host response that will be changed upon caspofungin treatment. Since caspofungin alters the composition of the fungal cell wall, it will also alter the pathogen associated molecular patterns (PAMPs) exposed on fungal cells. This alteration in PAMPs could result in a changed production and

expression of other signalling molecules, e.g. cytokines and chemokines, contributing to the process of fungal killing. It is expected that killing of fungal cells is achieved by caspofungin treatment in combination with the immune system by using direct and indirect signalling molecules including chitinases. However, the combined action of signalling molecules and mediators with chitinases needs to be elucidated in the future.

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CHAPTER 5

Polymorphisms altering chitotriosidase and AMCase activity do not determine the risk for Invasive Pulmonary Aspergillosis (IPA)

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Manuscript submitted

ABSTRACT

Chitin is a major fungal cell wall component and the human host produces chitinases chitotriosidase and AMCase upon fungal exposure in order to clear the fungus. Previously, many polymorphisms were described that influence the activity of these chitinases. We investigated the association between 4 of these polymorphisms in the genes encoding for chitotriosidase and AMCase and the incidence of IPA in an immunocompromised population. Four polymorphisms involved in the chitinase activity were studied in 89 subjects at risk for the development of IPA. These included a 24-bp insertion in the chitotriosidase gene (rs3831317), resulting in impaired chitinase activity and single nucleotide polymorphism (SNP) in the AMCase gene (rs61756687), resulting in decreased AMCase activity. Also, a SNP (rs41282492) and a 10-bp insertion in the 5'UTR region of the AMCase gene (rs143789088) were studied, both resulting in increased AMCase activity. DNA was isolated from blood and genotypes were determined using PCR-RFLP. According to clinical, radiological and mycological criteria, 18 patients developed proven or probable IPA and 71 subjects were controls.

No association with increased or decreased rate of aspergillosis was found with any of the polymorphisms ($p>0.05$). Also, classification of patients and controls based on theoretical chitinase activity could not prove an association between decreased chitinase activity and an increased risk for developing IPA. In conclusion, the four polymorphisms investigated here were not associated with increased or decreased risk for IPA.

INTRODUCTION

Invasive Pulmonary Aspergillosis (IPA) is a life-threatening infection caused mainly by the opportunistic fungal pathogen *Aspergillus fumigatus*. The incidence of IPA infection has increased over the last decades due to a rise in the use of immunosuppressive and immune modulatory drugs (1). Although almost all IPA patients are immunocompromised, only a small minority of immune compromised patients develops an invasive fungal infection. The question arises which underlying factors are involved in the development of IPA in these immunosuppressed patients.

One hypothesis is that the genetic make-up of the patient's immune system plays a role. The innate immune system is involved in the recognition of pathogens. Mammalian cells express several pattern recognition receptors (PRRs) to sense pathogens. PRRs involved in fungal sensing include toll-like receptor (TLR)-2, TLR-3, TLR-4, TLR-6 and TLR-9 and C-type lectin receptors dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), dectin-1 and dectin-2 (2-4). These PRRs sense fungal cell wall structures and pathogen-associated molecular patterns (PAMPs) expressed on the fungal cell wall, including mannan, β-glucan and nucleic acids (2, 4). Recognition of PAMPs results in activation of a signaling pathway and in the release of pro-inflammatory cytokines such as TNF-α and INF-γ, and of anti-inflammatory cytokines such as IL-4 and IL-10 (2). In previous reports, it was shown that polymorphisms in the genes for dectin-1, DC-SIGN (3), TNF receptor 1 (5) and in the IL-10 promoter gene (6) can lead to increased susceptibility to aspergilli in immune compromised patients.

Whether chitin is recognized as a PAMP is not known yet, however it is considered a possibility (2, 7). Chitin is an abundant polysaccharide that is found in fungi, insects and in nematodes and it is cleaved by chitinases. Chitin is the main cell wall component of the fungal cell wall. Although mammals do not produce chitin, they are capable of producing chitinases (8), in general in reaction to exposure to chitin. Two mammalian chitinases have been described: acidic mammalian chitinase (AMCase) and chitotriosidase.

In previous work, we described that both chitinases were increased in rats with invasive pulmonary aspergillosis (IPA)(9). We also described that both AMCase and chitotriosidase were necessary to degrade chitin after treatment with caspofungin (9). Hence we hypothesized that malfunction of the chitinases will impair the clearance of fungal material from the lungs and patients thus would be more prone to fungal infections.

Several genetic polymorphisms that influence chitinase activity in the human host were described. The most commonly described is a 24-bp insertion in the chitotriosidase gene (10-12), resulting in enzymatic inactivity in case of homozygosity. It is found in circa 6% of the Caucasian population (11). For the AMCase gene, several polymorphisms were described with an effect on the enzyme activity (13, 14). We hypothesized that

polymorphisms in the genes for chitinases, related to a reduced chitinase activity, were associated with a higher risk for the development of IPA. To test this hypothesis, four polymorphisms in either AMCase or chitotriosidase were determined in 86 patients with hematologic diseases, at risk for the development of IPA. The allele frequencies of these SNPs were compared between the patients who did develop IPA and those who did not.

MATERIALS & METHODS

Study population

Patients were included between November 2002 and April 2006 at Erasmus University Medical Center (Rotterdam, The Netherlands). Hospitalized adult patients with hematologic disease were eligible to participate in this study. Inclusion criteria were individuals who were at risk for prolonged neutropenia (neutrophil count < 500 cells/mm^3 for at least10 days), based on stem cell transplantation or chemotherapy. Exclusion criteria were evidence of fungal infection in lungs or sinuses, diagnosis of pneumonia at the time of study inclusion or a life expectancy of less than three months. Further details were described elsewhere (15). Patients who received prophylaxis with amphotericin B were excluded from the study. From all included subjects, blood samples were drawn routinely. Whole blood was stored at −80°C until processing. DNA was isolated from whole blood of these subjects using the MagNA Pure LC DNA Isolation kit - Large Volume (Roche Diagnostics Nederland BV, Almere, the Netherlands) according to the manufacturer's instructions. DNA was stored at −20°C until processing.

The study was approved by the medical ethical committee at Erasmus MC. Written informed consent was obtained according to guidelines from medical ethical committee at Erasmus MC.

Patients were assessed for fungal infection, according to international guidelines (16). When a fungal infection was suspected, patient material such as sputum, blood or broncho-alveolar lavage (BAL) fluid was cultured on Sabouraud's agar at 37°C for 96 hours, according to routine procedures (17). "Proven" or "probable" aspergillosis was defined according to the definition by the European Organization for Research and the Treatment of Cancer-Mycoses Study Group (EORTC-MSG)(18). In short, subjects were diagnosed with IPA when there was a host factor (e.g. prolonged neutropenia), a radiological sign of fungal disease (e.g halo sign, air-crescent sign) and a mycological factor (e.g. positive galactomannan index, positive culture). Patients lacking a mycological factor were classified as "possible" aspergillosis, according to the definitions. These patients were excluded from the analysis.

Genotyping

We searched PubMed Library for publications describing polymorphisms in the chitotriosidase gene and in the AMCase gene. Only polymorphisms causing an alteration (either increase or decrease) of the chitinase enzymatic activity were included. All PCR primers and amplification conditions are shown in Table 1. Restriction enzymes are also shown in Table 1. All enzymes were purchased at Fermentas and were used according to manufacturer's guidelines. Genotypes were determined by polymerase chain reaction restricted fragment length polymorphism (PCR-RFLP). PCR products were run at a 2.5% metaphor gel in 90 minutes. Two different persons assessed genotypes separately.

Table 1. PCR conditions for the different polymorphisms

Polymorphism	Primer sequence (5′ → 3′)	PCR program	Restriction endonuclease	Activity	Length (bp)	Ref
Chitotriosidase 24 bp insertion rs 3831317	F: agctatctgaagcagaag R: ggagaagccggcaaagtc	4′ 94°C + 40× (30″ 94°C + 30″ 55°C + 30″ 72°C) + 7′ 72°C	None	Normal Decreased	124 bp 148 bp	(11, 19, 20)
AMCase A50G rs 61756687	F: gtctcaccctgccttctttg R: acccaattctcctcggaaag	4′ 94°C + 40× (30″ 94°C + 30″ 58°C + 30″ 72°C) + 7′ 72°C	ApoI (XapI)	Normal Decreased	175 + 91 266	(21)
AMCase A290G rs 41282492	F: ctctgcctaccagctgacat R: gccattccgcaccgtataca	4′ 94°C + 40× (30″ 94°C + 30″ 58°C + 30″ 72°C) + 7′ 72°C	TaqI	Normal Increased	256 + 81 + 69 256 + 150	(14)
AMCase 10-bp insertion 5′UTR rs 143789088	F: ctgaccacagtatctaaacag R: ctgaccacagtatctaaacag	4′ 94°C + 40× (30″ 94°C + 30″ 58°C + 30″ 72°C) + 7′ 72°C	BfaI	Normal Increased	392 + 59 308 + 94 + 59	(13)

Statistical analysis

We used Pearson's χ^2 test to verify the Hardy-Weinberg equilibrium. Differences in allele frequencies were evaluated using Fisher's exact test (GraphPad Prism Software, San Diego, USA). A p-value of $p < 0.05$ was considered significant.

RESULTS

Blood samples were taken from 173 subjects in total. 84 subjects received Ambisome prophylaxis and were thus excluded from our study. The remaining 89 subjects were included and were genotyped. Of these 89 subjects, 15 subjects met the criteria for "proven" or "probable" aspergillosis and were classified as "patients". 3 subjects had "possible" aspergillosis and were excluded from the analysis. The remaining 71 subjects were classified as "controls".

Genotypes frequencies can be found in Table 2. The genotype distributions for all SNPs were consistent with the Hardy Weinberg equilibrium for both controls and patients.

Table 2. Distribution of insertion and SNPs in the genes for chitotriosidase and for AMCase.

Gene and genotype	Enzyme activity*	Patients (%) N=15	Controls (%) N=71	HWI** p-value	p-value
Chitotriosidase				0.177	1.000
Wildtype	Normal	8 (53.3%)	39 (54.9%)		
Heterozygous 24-bp insertion	Normal	7 (46.7%)	30 (42.3%)		
Homozygous 24-bp insertion	Impaired	0 (0%)	2 (2.8%)		
AMCase A50G				0.320	0.576
AA	Normal	12 (80.0%)	51 (71.8%)		
AG	Normal	3 (20.0%)	17 (23.9%)		
GG	Decreased	0 (0%)	3 (4.2%)		
AMCase A290G				0.326	0.419
AA	Normal	12 (80.0%)	47 (66.2%)		
AG	Normal	3 (20.0%)	23 (32.4%)		
GG	Increased	0 (0%)	1 (1.4%)		
AMCase 10-bp insertion 5'UTR				0.320	0.740
Wildtype	Normal	13 (86.7%)	56 (78.9%)		
Heterozygous 10-bp insertion	Normal	2 (13.3%)	15 (21.1%)		
Homozygous 10-bp insertion	Increased	0 (0%)	0 (0%)		

*Genotype associated with an impaired, normal or decreased enzyme activity according to previous publications (11, 13, 14, 19-21).

**Hardy Weinberg Equilibrium (HWE) as assessed by Pearson's χ^2 test. A p-value of >0.05 was associated with equilibrium.

The 24-bp insertion in the chitotriosidase gene is not associated with the development IPA

In the gene encoding for chitotriosidase, a 24-bp insertion was described previously (10, 11, 19, 20). The activity of chitotriosidase is reduced when a patient is found homozygous for this insertion (10, 22). Among the patients, 0/15 (0%) were homozygous for the 24-bp insertion and 7/15 (46.7%) were heterozygous. Among the controls, 2/71 (2.8%) were homozygous and 30/71 (42.3%) were heterozygous. The difference in allele frequency was not statistically significant (p = 1.00). Based on these data, being homozygous for the 24-bp insertion on the chitotriosidase gene was not associated with an increased risk for IPA.

Neither the A50G and A290G SNP, nor the 10-bp insertion in the 5'UTR region of AMCase is associated with IPA development

Previously, several SNPs in the AMCase gene were described, resulting in either increased or decreased AMCase activity (13, 14, 21). As shown in Tables 2, we studied two SNPs (A50G and A290G) and one 10-bp insertion in the 5'UTR region of the AMCase gene.

Allele frequencies of A50G and A290G in the patients were statistically similar to those in the controls (p = 0.42 and p = 0.58, respectively). Furthermore no difference was found in the presence of the 10-bp insertion between patients and controls (p = 0.74).

Prevalence of genotypes for increased or decreased chitinase is similar in patients and controls

From above results we could conclude that in this cohort of patients, a single polymorphism resulting in inactivity of a single chitinase, did not result in an increased prevalence of IPA. In previous work, we described that a combination of AMCase and chitotriosidase was necessary to degrade the fungal cell wall *in vitro* (9). We therefore also investigated whether a combination of polymorphisms, resulting in impaired

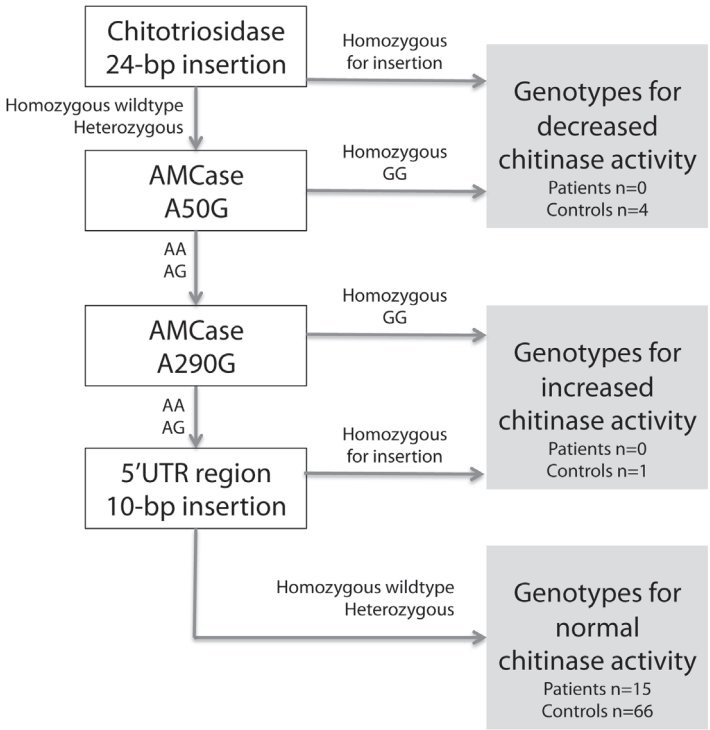

Figure 1. Flowchart in order to classify subjects as normal, increased or decreased chitinase activity, based on genotype of the 24-bp insertion for chitotriosidase and the polymorphisms and insertions for AMCase.

activity of either chitotriosidase or AMCase, was more prevalent in IPA patients than in the controls. We made the following assumptions: a subject who had hypothetically decreased activity of either chitotriosidase, AMCase or both had an elevated risk for developing IPA. A subject who had hypothetically normal chitotriosidase activity and increased AMCase activity had a decreased risk for developing IPA. We thus divided the patients and the controls each into three groups (see also Figure 1). The subjects in the first group with a hypothetically decreased chitinase activity had either a homozygous 24-bp insertion or a homozygous G-allele in the A50G SNP. The subjects in the second group with a hypothetically increased chitinase activity had no homozygous 24-bp insertion, no homozygous G-allele in the A50G SNP, and either a homozygous G-allele in the A290G SNP or a homozygous 10-bp insertion in the 5'UTR region. The third group with a hypothetically normal chitinase activity had no homozygous 24-bp insertion, no homozygous G-allele in the A50G SNP, no homozygous G-allele in the A290G SNP and no homozygous 10-bp insertion in the 5'UTR region. Based on this classification, all patients had the genotype for normal chitinase activity. No patient had the genotype for increased or decreased chitinase activity. 4/71 controls (5.6%) had the genotype for decreased chitinase activity, 66/71 (93.0%) had the genotype for normal chitinase activity and 1/71 (1.4%) had the genotype for increased chitinase activity. The prevalence of genotypes for decreased chitinase activity was similar between controls and patients ($p = 1.00$), as was the prevalence of genotypes for increased chitinase activity ($p=1.00$). Therefore, a genotype for decreased chitinase activity was not significantly correlated with an increased or decreased risk for IPA in this cohort of patients. Also, a genotype for increased chitinase activity was not proven to be protective for IPA.

DISCUSSION

In our previous study we showed that the immunocompromised host still produces chitinases in reaction to fungal exposure (9). Invasive pulmonary infection with *Aspergillus fumigatus* upregulated the production of chitotriosidase and AMCase in the lung. We showed that a combination of two chitinases and caspofungin could lyse the fungal cell wall. We hypothesized that chitinases are needed in the clearance of fungal material and that impaired chitinase activity or production is a risk factor for IPA in immunocompromised patients.

This impaired chitinase activity could be caused by the genetic make-up of the individual. In humans, we already know that SNPs in genes encoding for several PRRs such as TLR-2, TLR-3, TLR-4, TLR-6, TLR-9, DC-SIGN, dectin-1 and dectin-2 are associated with an increased risk for developing IPA. For both chitotriosidase and AMCase, insertions and SNPs were described which altered the enzyme activity. For chitotriosidase,

it was described that the carriers of the 24-bp insertion had a broad range of reduced activity (11, 19, 20). We thus considered heterozygotes of the 24-bp insertion as having normal chitotriosidase activity. We hypothesized that SNPs in the genes encoding for chitotriosidase and AMCase, resulting in an altered enzyme activity (13, 14, 21), were associated with the risk for developing IPA. In our study, we could not demonstrate an association between individual polymorphisms in the genes resulting in decreased chitinase activity and the incidence of IPA. Also the polymorphisms resulting in increased chitinase activity were not found to be associated with a decreased risk for developing IPA. This was unexpected, but since our cohort of patients is of limited size, the lack of association could be caused by a lack of statistical power. It is very difficult to obtain a large enough study population of IPA patients due to the limited availability of patients with IPA and the heterogeneity of the study population. Furthermore, patients with IPA are missed since the diagnosis of IPA is often difficult or empirical therapy is given before the diagnosis is established with certainty. Therefore it is not surprising that only few cohorts of patients with invasive pulmonary fungal have been described that investigated genetic polymorphisms as risk factor for IPA (3, 23-31). Enlarging the cohort by including patients from other geographical areas is prone to bias, since from other studies it was already demonstrated that the allele frequency differs in different geographical areas (10, 22, 32). In our population, homozygosity for the insertion was found in 2.8% of the mainly European Caucasian controls, which is lower than the 6% that was reported previously in European Caucasians (11). Allele frequencies could be different in certain populations or patient groups. Our population was at risk for prolonged neutropenia and the prevalence of the insertion might be different in this particular population.

Finally, even though we could not prove that the described polymorphisms were associated with increased incidence of IPA, there might be an association with a more severe clinical course or a worse outcome of the infection. However this was not investigated in our study.

Summarizing, the SNPs and insertions in the genes for chitotriosidase and AMCase that were investigated here, did not show a significant association with the development of IPA. Clearly IPA may develop in individuals with genotypes associated with normal chitinase activity. Apparently, these genotypes by themselves do not determine the susceptibility of patients for IPA, although they may, in a much larger study, still be found to contribute to the risk. Other factors of the human immune system may play a role as well, such as different polymorphisms in other genes involved in the recognition of PAMPs. More studies could increase the insight in the genetic risk factors for the development of IPA.

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CHAPTER 6

A polymorphism in the chitotriosidase gene associated with risk of mycetoma due to *Madurella mycetomatis* mycetoma – a retrospective study

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PLoS Negl Trop Dis 2015;9(9):e0004061

ABSTRACT

Madurella mycetomatis is the most prevalent causative agent of eumycetoma in Sudan, an infection characterized by the formation of grains. Many patients are exposed to the causative agent, however only a small number develop infection. *M. mycetomatis* contains chitin in its cell wall, which can trigger the human immune system. Polymorphisms in the genes encoding for the chitin-degrading enzymes chitotriosidase and AMCase were described, resulting in altered chitinase activity. We investigated the association between 4 of these polymorphisms and the incidence of *M. mycetomatis* mycetoma in a Sudanese population.

Polymorphisms studied in 112 eumycetoma patients and 103 matched controls included a 24-bp insertion in the chitotriosidase gene (rs3831317), resulting in impaired chitinase activity and single nucleotide polymorphism (SNP) in the AMCase gene (rs61756687), resulting in decreased AMCase activity. Also, a SNP (rs41282492) and a 10-bp insertion in the 5'UTR region of the AMCase gene (rs143789088) were studied, both resulting in increased AMCase activity. DNA was isolated from blood and genotypes were determined using PCR-RFLP.

Histological staining proved the presence of chitin in the fungal grain. The polymorphism resulting in decreased chitotriosidase activity was associated with increased odds of eumycetoma (odds ratio 2.9; p=0.004). No association was found for the polymorphisms in the genes for AMCase (all p>0.05). In conclusion, decreased chitotriosidase activity was associated with increased risk of *M. mycetomatis* mycetoma.

INTRODUCTION

Mycetoma is a chronic infectious granulomatous disease that is frequently reported in tropical and subtropical climates between 30°N and 15°S of the equator. Mycetoma can be caused by bacteria (actinomycetoma) and fungi (eumycetoma), though the fungus *Madurella mycetomatis* is the most common causative agent in the world (1). In Sudan it accounts for over 70% of all mycetoma cases (1). The lower extremities are affected most, though other sites of the body can be affected as well (1). After traumatic inoculation of the causative agent into the subcutaneous tissue, usually in the sole of the foot, the disease progresses and invades the deep structures and the skin. Multiple nodules and sinuses discharging pus are formed and grains develop. In the grains, the fungal mycelium is embedded in hard brown matrix containing melanin. This grain is thought to protect the fungus from the host immune system.

In endemic areas in Sudan, *M. mycetomatis* DNA was found in the soil (2) and antibodies against mycetoma causative agents have been detected in the majority of the inhabitants in these areas (3). However, it is not clear why only a minority of the exposed humans develop overt clinical infection. Several environmental and patient-related factors have been described to influence the risk for the development of mycetoma, including concurrent schistosomiasis (4). Furthermore, several associations with genetic polymorphisms in genes involved in hormone synthesis (5) and parts of the immune system, including collagenases and gelatinases were reported (3, 6, 7). However, the role of chitinases in eumycetoma caused by *M. mycetomatis* was not investigated previously.

Infection with *M. mycetomatis* often results in fungal grain development in the tissue. The exact composition of the grain is not known. Ibrahim *et al* reported that the grains contain melanin, heavy metals, proteins and lipids, resulting in a cement matrix in which the mycelium is embedded (8). Probably, the grain also contains chitin, since chitin is one of the cell wall components of many fungi. Around the fungal grain, the host's innate immune system mounts an inflammatory response, resulting in a reactive granuloma laden with neutrophils, macrophages and other inflammatory cells (9). Chitinases are part of the innate immune system and, in general, their production by macrophages and other cells is upregulated by the host's tissue exposure to chitin. Chitinases seem to play a role in allergic and infectious diseases caused by chitin-bearing organisms (10-12), however the exact role of these enzymes in pathogenesis is yet to be elucidated. Chitinases hydrolyze chitin, which is the main component of the fungal cell wall. Decreased chitinase activity could, therefore, result in an enhanced susceptibility towards infections by chitin bearing fungi. Currently, two true chitinases are known in humans: chitotriosidase and acidic mammalian chitinase (AMCase)(13). Polymorphisms in the genes for these chitinases have been described that are associated with either

increased or decreased enzyme activity (12, 14-17). Impaired or decreased activity of either one or both of these chitinases could result in increased susceptibility towards fungal infections including eumycetoma. The aim of this study is to investigate the role of chitinase activity in the development of mycetoma caused by *M. mycetomatis*. We hypothesized that chitotriosidase and AMCase polymorphisms, resulting in decreased chitinase activity, would be found more frequently in mycetoma patients than in controls.

MATERIALS & METHODS

Study population

Individuals presenting at Mycetoma Research Center, Khartoum between 2001 and 2008, were eligible for inclusion, when the diagnosis of *Madurella mycetomatis* mycetoma was confirmed. People living in the same endemic regions were included as controls. The demographic features are given in Table 1. Retrospectively, genotypes were determined of 112 *Madurella mycetomatis* infected patients and 103 healthy endemic controls, matched for sex and age. Whole blood was stored at −80°C until processing. DNA was isolated from whole blood of these subjects using the MagNA Pure LC DNA Isolation kit - Large Volume (Roche Diagnostics Nederland BV, Almere, the Netherlands) according to the manufacturer's instructions. DNA was stored at −20°C until processing. Tissue of the foot and of the grain was obtained in 1998 from Sudanese subjects, infected with *M. mycetomatis*.

Table 1. Study population demographic features.

Characteristic		Mycetoma patients (n=112)	Endemic controls (n=103)
Gender (male/female)		79/33	77/26
Mean duration in years (range)		6.9 (1-27)	N/A
Mycetoma lesion site*	Foot	87	N/A
	Hand	12	N/A
	Lower leg	14	N/A
Mycetoma lesion size	Small (<5 cm)	55	N/A
	Moderate (5-10 cm)	20	N/A
	Massive (>10 cm)	38	N/A

* One patient had two lesions, one of the foot and one of the hand

Genotyping

PubMed Library was searched for publications describing polymorphisms in the chitotriosidase gene and in the AMCase gene. We included only polymorphisms that were described elsewhere to result in an alteration (either increase or decrease) of the chitinase enzymatic activity. PCR primers, amplification conditions and restriction enzymes used in this study are shown in Table 2. We purchased all enzymes at Fermentas (Thermo Fisher Scientific, Waltham, USA). Enzymes were used according to manufacturer's guidelines. We determined genotypes by polymerase chain reaction restricted fragment length polymorphism (PCR-RFLP). PCR products were run at a 2.5% metaphor gel in 90 minutes. Two different investigators assessed genotypes separately.

Table 2. PCR conditions for the different polymorphisms.

Polymorphism	Primer sequence (5'→3')	PCR program	Restriction endonuclease	Activity	Length (bp)	Ref
Chitotriosidase 24-bp insertion (rs 3831317)	F: agctatctgaagcagaag R: ggagaagccggcaaagtc	4' 94°C + 40× (30" 94°C + 30" 55°C + 30" 72°C) + 7' 72°C	None	Normal Decreased	124 148	(14, 19, 20)
AMCase A50G (rs 61756687)	F: gtctcaccctgccttctttg R: acccaattctcctcggaaag	4' 94°C + 40× (30" 94°C + 30" 58°C + 30" 72°C) + 7' 72°C	ApoI (XapI)	Normal Decreased	175 + 91 266	(21)
AMCase A290G (rs 41282492)	F: ctctgcctaccagctgacat R: gccattccgcaccgtataca	4' 94°C + 40× (30" 94°C + 30" 58°C + 30" 72°C) + 7' 72°C	TaqI	Normal Increased	256 + 81 + 69 256 + 150	(17)
AMCase 10-bp insertion 5'UTR (rs 143789088)	F: ctgaccacagtatctaaacag R: ctgaccacagtatctaaacag	4' 94°C + 40× (30" 94°C + 30" 58°C + 30" 72°C) + 7' 72°C	BfaI	Normal Increased	392 + 59 308 + 94 + 59	(16)

Immunohistochemistry

Tissue biopsies were fixed in formalin, embedded in paraffin and processed for immunohistochemical evaluation. To determine if the antibodies directed against human AMCase and chitotriosidase did not cross-react with any fungal proteins, heat-fixed *in vitro* grown *M. mycetomatis* hyphae were stained with the same protocol. Histologic sections (coupes) were deparaffinised in xylene, then rehydrated in decreasing concentrations of ethanol. Haematoxylin and eosin staining was used as standard staining. Calcofluor-white staining was used to visualize chitin, according to manufacturer's guidelines. Coupes were washed in aquadest and incubated with 25 µM calcofluor-white (Molecular Probes®, Leiden, The Netherlands) for 30 minutes at 37°C in the dark. Afterwards, coupes were washed in aquadest and assessed by fluorescent microscopy.

Presence of chitinase was determined as described previously (10). First, endogenous peroxidase was blocked in methanol with 0.3% H_2O_2 and non-specific binding sites were blocked with rabbit or goat serum. Subsequently, coupes were incubated overnight with rabbit polyclonal antibody directed against chitotriosidase (H-66, 1:75, Santa Cruz Biotechnology, Santa Cruz, USA) or with goat polyclonal antibody directed against AM-Case (Y-14, 1:50, both Santa Cruz Biotechnology, Santa Cruz, USA). As a control, a goat polyclonal IgG antibody was used, directed against swine IgM (A100-100A, 1:50, Bethyl Laboratories, Montgomery, USA). From the VectaStain® Elite ABC kit (Vector Laboratories Burlingame, CA, USA), we used anti-rabbit IgG or anti-goat IgG as a secondary antibody and the coupes were developed using the protocol from the kit. Haematoxylin was used as counter staining.

Ethics statement

Written informed consent was obtained from patients and controls, according to guidelines from the medical ethical committee at Soba University Hospital, Khartoum, Sudan. The study protocol was approved by this medical ethical committee.

Statistical analysis

Pearson's χ^2 test was used to verify the Hardy-Weinberg equilibrium. Differences in allele frequencies were determined using the two-sided Fisher's exact test (GraphPad Prism Software, San Diego, USA). A p-value of $p < 0.05$ was considered significant.

RESULTS

Mycetoma grains caused by *Madurella mycetomatis* contain chitin and chitinase

Tissue sections of the fungal grain caused by *M. mycetomatis* are shown in Figure 1. The sections were stained using haematoxylin and eosin (HE) (Figure 1A), grocott's methenamine silver stain (Figure 1B) and calcofluor-white stain (Figure 1C). In Figure 1A it is clearly seen that the grain itself contains cement material. The hyphae are embedded within this cement material (Figure 1B). In Figure 1C, fungal chitin is stained by calcofluor-white (18). This figure shows that the chitin is mainly found in the hyphae inside the grain. The cement material itself is not stained. Since chitin is present in the hyphae inside the grain, we hypothesized that the host produces chitinases, in reaction to exposure to chitin. Thus, tissue sections were stained for chitotriosidase and for AMCase (Figure 2). The presence of chitinases is shown by a red color, which was mainly located in the grain on the fungal hyphae and not in the cement component of the grain. This seems consistent with the presence of the chitin itself, which was also mainly found on the fungal hyphae and not in the cement material. We also found that both

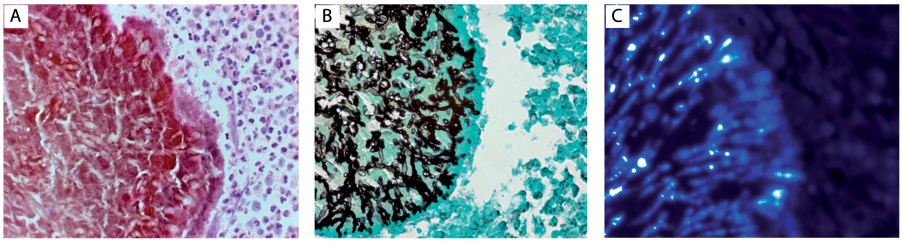

Figure 1. Tissue sections of Mycetoma foot, showing the fungal grain. Magnification 400×. A. Haematoxylin and eosin staining. The grain, consisting of cement and fungal hyphae, is colored red. Around the grain, a zone with neutrophils is visible.
B. Grocott's methenamine silver staining. The hyphae inside the grain are stained black. C. Calcofluor white staining. Chitin is stained by calcofluor white staining (18). This photo illustrates that hyphae inside the grain are stained, and not the cement component of the grain.

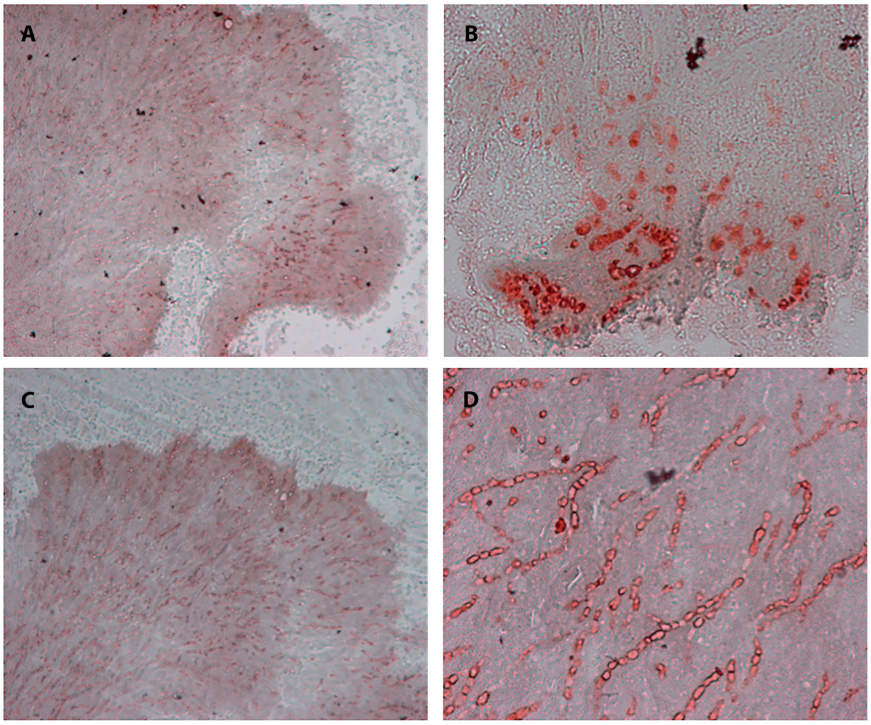

Figure 2. Tissue sections of Mycetoma foot stained for chitotriosidase and for AMCase. Magnification ×100 (A and C) and ×400 (B and D).
A and B: Chitotriosidase.
C and D: AMCase.
Presence of both enzymes is shown by red color. The grain is clearly visible and colored red diffusely. Inside the grain, fungal hyphae are stained more intensely, showing an increased presence of chitotriosidase and AMCase around the fungal hyphae.

chitotriosidase and AMCase were found in the tissue surrounding the fungal grain, however both chitinases concentrated in the grain. The staining reaction for chitotriosidase was specific, since no binding of the chitotriosidase specific antibodies was noted on *in vitro* grown fixated *M. mycetomatis* hyphae. In contrast, binding of the AMCase specific antibodies was noted on some *in vitro* grown, fixated *M. mycetomatis* hyphae, although the staining was less intense than that inside the grain. Therefore it is plausible that not only chitotriosidase but also AMCase are indeed binding to the hyphae inside the mycetoma grain.

Polymorphisms in the genes for chitinases cause a risk for infection with *M. mycetomatis*

We subsequently investigated whether genetic polymorphisms that resulted in either increased or decreased activity of either chitinase, were associated with an increased risk for invasive fungal mycetoma disease. Exactly 215 Sudanese subjects were included in the study and were genotyped. Of these, 112 subjects had active mycetoma disease and were classified as patients, and 103 subjects were healthy controls. Genotype frequencies are shown in Table 3.

Table 3. Distribution of polymorphisms in the genes for chitotriosidase and for AMCase.

Gene polymorphism	Genotype	Enzyme activity*	Patients (%) n=112	Controls (%) n=103	HWE** p-value	p-value	Odds ratio (95% CI interval)
Chitotriosidase 24-bp insertion	Wildtype	Normal	84 (75%)	94 (91%)	0.106	0.004	2.9 (1.4-6.1)
	Heterozygous 24-bp insertion	Decreased	27 (24%)	8 (8%)			
	Homozygous 24-bp insertion	Impaired	1 (1%)	1 (1%)			
AMCase A50G	AA	Normal	92 (82%)	83 (81%)	0.940	0.647	1.1 (0.7-1.8)
	AG	Normal	14 (13%)	19 (18%)			
	GG	Decreased	6 (5%)	1 (1%)			
AMCase A290G	AA	Normal	74 (66%)	67 (65%)	0.657	0.717	1.2 (0.6-2.1)
	AG	Normal	30 (27%)	33 (32%)			
	GG	Increased	8 (7%)	3 (3%)			
AMCase 10-bp insertion 5'UTR	Wildtype	Normal	73 (65%)	66 (64%)	0.578	0.720	1.1 (0.7-1.8)
	Heterozygous 10-bp insertion	Normal	31 (28%)	34 (33%)			
	Homozygous 10-bp insertion	Increased	8 (7%)	3 (3%)			

*Genotype associated with an impaired, normal or decreased enzyme activity according to previous publications (14, 16, 17, 19-21).

**Hardy Weinberg Equilibrium (HWE) as assessed by Pearson's χ^2 test. A p-value of >0.05 was associated with equilibrium.

The genotype distribution for all polymorphisms was consistent with the Hardy Weinberg equilibrium.

The 24-bp insertion in the chitotriosidase gene is associated with invasive mycetoma

In the gene encoding for chitotriosidase, a 24-bp insertion in exon 10 was described previously (12, 14, 19, 20). It was described elsewhere that chitotriosidase activity is reduced when patients are heterozygous for this insertion and completely absent when they were homozygous for this allele (12, 14). Among the patients, 1/112 (0.9%) was homozygous for the 24-bp insertion and 27/112 (24.1%) were heterozygous. Among the controls, 1/103 (1.0%) was homozygous for the insertion and 8/103 (7.8%) were heterozygous. The insertion containing allele was found significantly more frequently in the patients than in the control group (p=0.004). Based on these data, the 24-bp insertion in the chitotriosidase gene does increase the risk for invasive mycetoma disease caused by *M. mycetomatis* (odds ratio 2.9; 95% CI 1.4 – 6.1).

Neither the A50G and the A290G SNP, nor the 10-bp insertion in the 5'UTR region of AMCase is associated with invasive mycetoma

Several SNPs in the AMCase genes were described previously, resulting in either increased or decreased AMCase activity (16, 17, 21). As shown in Table 2, we studied two SNPs (A50G and A290G) and one 10-bp insertion in the 5'UTR region of the AMCase gene. Allele frequencies of A50G and A290G in the patients were similar to those in the controls (p=0.65; odds ratio 1.2; 95% CI 0.6-2.1; and p=0.72; odds ratio 1.1; 95% CI 0.7-1.8, respectively). Furthermore, no difference was found in the presence of the 10-bp insertion between patients and controls (p=0.72; odds ratio 1.1; 95% CI 0.7-1.8).

DISCUSSION

Madurella mycetomatis is the most prevalent causative agent of eumycetoma worldwide and in Sudan in particular (1). Many inhabitants of Sudan are exposed to this causative agent, however few of them develop mycetoma. Currently, the predisposing factors for mycetoma are not known, but some genetic polymorphisms have been associated with the development of mycetoma (4, 6, 7).

In this paper, we first showed that chitin is present in the *M. mycetomatis* grain and that two human chitinases are found in the vicinity of this grain, in reaction to exposure to *M. mycetomatis* mycetoma. Both AMCase and chitotriosidase seemed to concentrate on the chitin-containing fungal hyphae, a phenomenon that was previously found in rats infected with *Aspergillus fumigatus* (10). Since in both mycetoma and in *A. fumigatus*

infected tissue AMCase and chitotriosidase concentrated on chitin-containing fungal hyphae, it needed to be determined if this was not due to cross-reaction of fungal proteins with the antibodies used. Staining *in vitro* grown *M. mycetomatis* hyphae with an antibody for chitotriosidase showed no staining on the hyphae. Also, no staining of *A. fumigatus* hyphae occurred (10), making it likely that the chitotriosidase antibodies were specifically directed against mammalian chitotriosidase and that fungi expressed no proteins which share epitopes with this enzyme. However, it should be kept in mind that *in vitro* grown fungi could express different proteins than could be expressed in a grain. In contrast to the specificity of the chitotriosidase antibody, the AMCase antibody seemed less specific. When stained with the AMCase antibody, some staining of the *in vitro* grown *M. mycetomatis* hyphae was noted. This was not the case for *A. fumigatus* hyphae (10). Apparently, a protein with an epitope similar to that of AMCase is located on some fungal hyphae when grown *in vitro*. Therefore the AMCase stained in the tissue samples of the patients could be the result of both expressed human AMCase and a protein of fungal origin. Since the staining was more intense in the tissue sections, we feel that it is likely that AMCase was indeed present.

Next to demonstrating that AMCase and chitotriosidase were present at the site of infection, we also provided evidence that a polymorphism in the gene for chitotriosidase, resulting in impaired enzyme activity, significantly increased the risk for the development of eumycetoma. Increased or decreased activity of the alternative human chitinase, AMCase, did not have a significant influence on the risk for eumycetoma. In *M. mycetomatis* mycetoma, chitotriosidase is apparently more crucial than AMCase. Although chitotriosidase and AMCase are both chitinases, the cleavage site of both chitinases differs. Chitotriosidase is an exochitinase, whereas AMCase is an endochitinase, referring to the site where the enzyme cleaves the chitin chain (22). Apparently, exochitinase activity is more important in the prevention of mycetoma than endochitinase activity. This is supported by the fact that in certain diseases an association was found with only one chitinase and not with both. In genetic association studies conducted in patients with bronchial asthma, an association was reported with only one chitinase, and not with both of them (21, 23, 24), indicating that both chitinases may have the same substrate, but have a distinct function in humans. Chitin and the produced chitotriosidase seem to be important in the development of the mycetoma grain. Since many mycetoma patients have the wild type chitinases associated with normal levels and activity of these enzymes, however, polymorphisms in these enzyme-encoding genes are clearly not the only factors that determine the risk for *M. mycetomatis* mycetoma.

Many previous reports showed that polarization of the immune response seems to play a role in the development of mycetoma (4, 7, 25-28). The development of mycetoma is associated with a Th2 response. Based on immunohistochemistry studies in various mycetoma causative agents, it appeared that the cytokine pattern surrounding the

mycetoma grain is a Th2 response. IL-10 and IL-4, both Th2-associated cytokines, were highly expressed around the fungal grain (7, 26, 27). A different study showed that after stimulation of peripheral blood mononuclear cells (PBMCs) with mycetoma antigens, a Th2 response developed in mycetoma patients and a Th1 response developed in healthy endemic controls (25). Indirect evidence for a Th2 response was also found in the association between schistosomiasis, associated with a Th2 response, and eumycetoma (4). Not only cytokines, but also other mediators matching Th2 response were reported in mycetoma.

Sandler *et al* (28) showed that mice with a Th2 response have increased expression of matrix metalloproteinases (MMPs) and of tissue inhibitor of MMP-1 (TIMP-1). Furthermore, AMCase was induced in Th2-polarized mice (28), which was confirmed by several other studies (29-31). Furthermore, Geneuglijk *et al* confirmed that MMP-2 and MMP-9 were expressed in the mycetoma lesion (7). In this paper we also demonstrated that AMCase and chitotriosidase are expressed in the mycetoma lesion.

In contrast to AMCase, which is induced in a Th2 response, chitotriosidase is produced in the environment of a Th1 response (32). In our study we showed that impaired function of chitotriosidase increases the risk to develop mycetoma.

Since Elagab *et al* already demonstrated that the PBMCs of healthy endemic controls produce Th1 cytokines when exposed to *M. mycetomatis* antigens, it is likely that they also produce high levels of chitotriosidase in order to eliminate *M. mycetomatis*. In individuals with a genotype resulting in impaired chitotriosidase activity, elimination of *M. mycetomatis* could be less efficient, leading to the development of a mycetoma lesion. More research is needed to unravel the exact role of the host in the development of the mycetoma grain.

Based on the data of this study and of other studies, we created the following hypothesis. When a host is exposed to *M. mycetomatis*, it will try to eliminate the pathogen. Most individuals will respond with a Th1 polarized response. In this response acute inflammation develops, chitotriosidase is produced and no grain is formed. When the response is mainly Th2-polarized, a chronic inflammatory process in which both chitotriosidase and AMCase are produced, results in the formation of granulomas with grains. The chronic exposure to fungal material in combination with the Th2 response also results in an increase in MMPs, which further modulates the collagen capsule surrounding the grain (2, 8, 28). Polymorphisms in the genes for IL-10, CCL-5 and TIMP-1 were shown to increase the risk for mycetoma (7, 8). Chitinases degrade the chitin component of the fungal cell wall (10). A polymorphism in the gene for chitotriosidase results in impaired enzyme activity (14), which in its turn results in an increased risk of developing *Madurella mycetomatis* mycetoma, as we showed in this paper.

In conclusion, in this study we demonstrated that the grain caused by *Madurella mycetomatis*, contains chitin. The human immune system produced both AMCase and chitotriosidase in the vicinity of this grain. Only the 24-bp insertion in the gene for chitotriosidase was associated with the development of mycetoma caused by *M. mycetomatis*.

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CHAPTER 7

Discrimination of *Aspergillus lentulus* from *Aspergillus fumigatus* by Raman spectroscopy and MALDI-TOF MS

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Eur J Clin Microbiol Infect Dis 2014;33(2):245-251

ABSTRACT

In 2005, a new sibling species of *Aspergillus fumigatus* was discovered: *Aspergillus lentulus*. Both species can cause invasive fungal disease in immunocompromised patients. The species are morphologically very similar. Current techniques for identification are PCR-based or morphology-based. These techniques are labour-intense and not sufficiently discriminatory. Since *A. lentulus* is less susceptible to several antifungal agents, it is important to correctly identify the causative infectious agent in order to optimize antifungal therapy. In this study we determined whether Raman spectroscopy and/or MALDI-TOF MS were able to differentiate between *A. lentulus* and *A. fumigatus*. For 16 isolates of *A. lentulus* and 16 isolates of *A. fumigatus*, Raman spectra and peptide profiles were obtained using the Spectracell and the MALDI-TOF MS (VITEK MS RUO, bioMérieux), respectively. In order to obtain reliable Raman spectra for *A. fumigatus* and *A. lentulus*, the culture medium needed to be adjusted to obtain colourless conidia. Only Raman spectra obtained from colourless conidia were reproducible and correctly identified 25 out of 32 (78%) of the *Aspergillus* strains. For VITEK MS RUO, no medium adjustments were necessary. Pigmented conidia resulted in reproducible peptide profiles as well in this case. VITEK MS RUO correctly identified 100% of the *Aspergillus* isolates, in a timeframe of approximately 54 hours including culture. Of the two techniques studied here, VITEK MS RUO was superior to Raman spectroscopy in the discrimination of *A. lentulus* from *A. fumigatus*. VITEK MS RUO seems to be a successful technique in the daily identification of *Aspergillus* species within a limited timeframe.

INTRODUCTION

Over the last few decades, immunosuppressive therapies have become increasingly available. As a result, the number of immunocompromised patients and of difficult-to-treat opportunistic infections among them, including invasive fungal infections, is still rising (1-4). Invasive pulmonary aspergillosis (IPA) is one of these difficult-to-treat opportunistic fungal infections. Several *Aspergillus* species are able to cause IPA in humans. The most prevalent species is *Aspergillus fumigatus*, macroscopically characterised by green-blue colonies on Sabouraud's dextrose agar and microscopically by septated hyphae with conidiophores (5). With the introduction of genetic identification it appeared that several fungi were misidentified as *A. fumigatus* in the past (6). One of these species was *Aspergillus lentulus* (7). Owing to its similar morphology, both macroscopically and microscopically, discrimination of both species remains complicated. This can be demonstrated by the fact that 16% of isolates, morphologically identified as *A. fumigatus*, appeared to be *A. lentulus*, based on molecular analysis (6). Since both species are not equally susceptible to the antifungal agents currently in use (amphotericin B, itraconazole, voriconazole and caspofungin (8-10)), it is important to discriminate these two species. Early detection and identification of the causative agent results in the early start of appropriate treatment. This will enhance the success rate of treatment and limits the costs of hospitalization (11).

Since the discrimination of *A. lentulus* from *A. fumigatus* based on morphologic characteristics is almost impossible, PCR and sequencing remain necessary. In many medical mycology laboratories these PCR-based techniques are not routine procedures, thus requiring a time-consuming approach. Therefore, it would be ideal to define an easy method of distinguishing between the two fungal species quickly and reliably. In recent years, new diagnostic strategies based on biophysics became available for the detection and identification of many types of pathogenic micro-organisms. These new platforms are less labour-intensive than the conventional identification techniques and have significantly higher resolution and significantly shortening the diagnostic process in many instances. Raman spectroscopy and matrix-associated laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) are two of these new techniques. Raman spectroscopy is a non-destructive optical technique, based on scattering of light by molecules. It generates a profile based on all cell components and was used successfully to identify bacterial species (12, 13). However, no procedure has as yet been suggested for identification of fungi with Raman spectroscopy. MALDI-TOF MS generates a limited profile based on masses and has been proven useful in the determination of fungi (14-16). However, identification of *A. lentulus* by MALDI-TOF MS was not described extensively. In the present study we used a group of well-characterised *A. lentulus* and

A. fumigatus isolates to determine whether either Raman spectroscopy or MALDI-TOF MS were able to discriminate these two *Aspergillus* species.

MATERIALS & METHODS

Fungal strains

A panel of 16 independently isolated *A. lentulus* clinical isolates was kindly provided by Janet Staab (John Hopkins University School of Medicine, Baltimore, MD, USA). The *A. lentulus* strains included were FH1, FH4, FH5, FH6, FH7, FH84, FH85, FH86, FH219, FH220, FH231, FH238, FH239, FH265, FH292 and FH293. These strains were confirmed to be *A. lentulus* based on multi-locus sequence typing (MLST) of five genes: the beta-tubulin gene, the rodlet A gene, the salt-responsive gene, the mitochondrial cytochrome b gene, and the internal transcribed spacer regions (7). The *A. fumigatus* strains were 39, 41, 44, 47, 293, D201-1, DP, k43, k52, k57, k65, k68, k74, k76, k80. These 15 heterogenic strains are all clinical *A. fumigatus* strains, collected at Erasmus MC, Rotterdam, the Netherlands. Based on typing of the CSP gene, they were found genetically divers and unrelated. Furthermore *A. fumigatus* ATCC 204305 was included as laboratory strain. Species identification was confirmed by ITS sequencing as previously described (5). Fungi were cultured on Sabouraud's dextrose agar (SDA; bioMérieux, Marcy l'Etoile, France) with or without 1% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Zwijndrecht, the Netherlands).

Raman spectroscopy

Strains were cultured for 96 hours at 37°C before conidia were harvested in phosphate buffered saline (PBS) containing 0.05% Tween (Sigma-Aldrich, Zwijndrecht, the Netherlands). The conidia were washed twice and suspended in sterile water (aquadest, bioMérieux) and spotted on a microslide carrier. Samples were allowed to dry for 15 min and Raman spectra were measured in the commercially available Spectracell*RA*® Bacterial Strain Analyzer (River Diagnostics, Rotterdam, the Netherlands). Results were analysed and dendrograms were created using the Spectracell*RA*® Bacterial Strain Analyzer software (version 1.7.8).

MALDI-TOF MS

Strains were cultured for 48 and 192 hours at 30°C. Fungal material was gently removed with a sterile swab, which was pre-moistened with medium suspension (bioMérieux, Ref. 20150), and suspended in medium suspension. Ethanol absolute (0.9 mL; Sigma-Aldrich, Lyon, France) was added, samples were mixed and centrifuged for 2 min at 15,000 × *g* and the supernatant was discarded. Next, 40 µl of 70% formic acid and 40 µl acetonitril (both Sigma-Aldrich, Lyon, France) were added and samples were mixed and

centrifuged for 2 min at 15,000 × *g*. The supernatant was spotted on a single-use target (VITEK-MSDS, bioMérieux, Marcy l'Etoile, France) and samples were allowed to dry. One microliter of α-cyano-4-hydroxycinnamic acid (CHCA; bioMérieux) was added as a matrix and targets were allowed to dry. Spectra were determined in MALDI-TOF MS AXIMA Assurance (VITEK MS RUO; Shimadzu, Champs-sur-Marne, France) in a linear positive ion extraction mode within a mass range from 2,000 to 20,000 Da. The system was calibrated externally with the mass spectrum obtained from fresh cells of *E. coli* ATCC 8739 strain. Spectra were automatically processed with the Launchpad V2.8. software (Shimadzu Biotech, Columbia, MD, USA). The resulting peak lists were exported and analysed using SARAMIS software (bioMérieux VITEK MS RUO) for spectra comparison. Clusters were produced by hierarchical agglomerative clustering using SARAMIS absolute or relative similarity measure and single-linkage criterion. Cluster analysis was performed by SARAMIS computing dendrograms based on similarities between masses.

Reproducibility was tested by sampling and measuring one strain of both fungal species in triplicate. Protein extracts were randomly performed on the 32 strains in order to prevent bias.

Turn around time

The time frames of different techniques were compared from the inoculation of the strains on the first culture medium. Included were culture time, process of the fungal material to measurable content, measurement of the fungal material and analysis of the data (e.g. cluster analysis).

RESULTS

Raman spectroscopy does not distinguish all *A. lentulus* isolates from *A. fumigatus* isolates

When analysing the *Aspergillus* species with Raman spectroscopy, we noted that the green pigment resulted in a strong signal, masking the original spectrum. Raman spectra of the triplicates were not reproducible. This problem was solved when the fungal isolates were incubated on SDA with 1% DMSO, resulting in white conidia (17). Obviously, when harvesting the conidia, most *A. lentulus* strains demonstrated a typically slowly sporulating phenotype. Nevertheless, enough conidia were produced to prepare samples for Raman spectroscopy. Raman spectra could be obtained for all 32 strains. As shown in Figure 1, the isolates separated in two major clusters, the first consisting of mainly *A. fumigatus* strains and the second consisting of mainly *A. lentulus* strains. However, six *A. fumigatus* strains fell into the *A. lentulus* cluster: *A. fumigatus* 39, k74, k76, k68, k80 and D201-1. One *A. lentulus* isolate, *A. lentulus* FH292, fell into the *A. fumigatus*

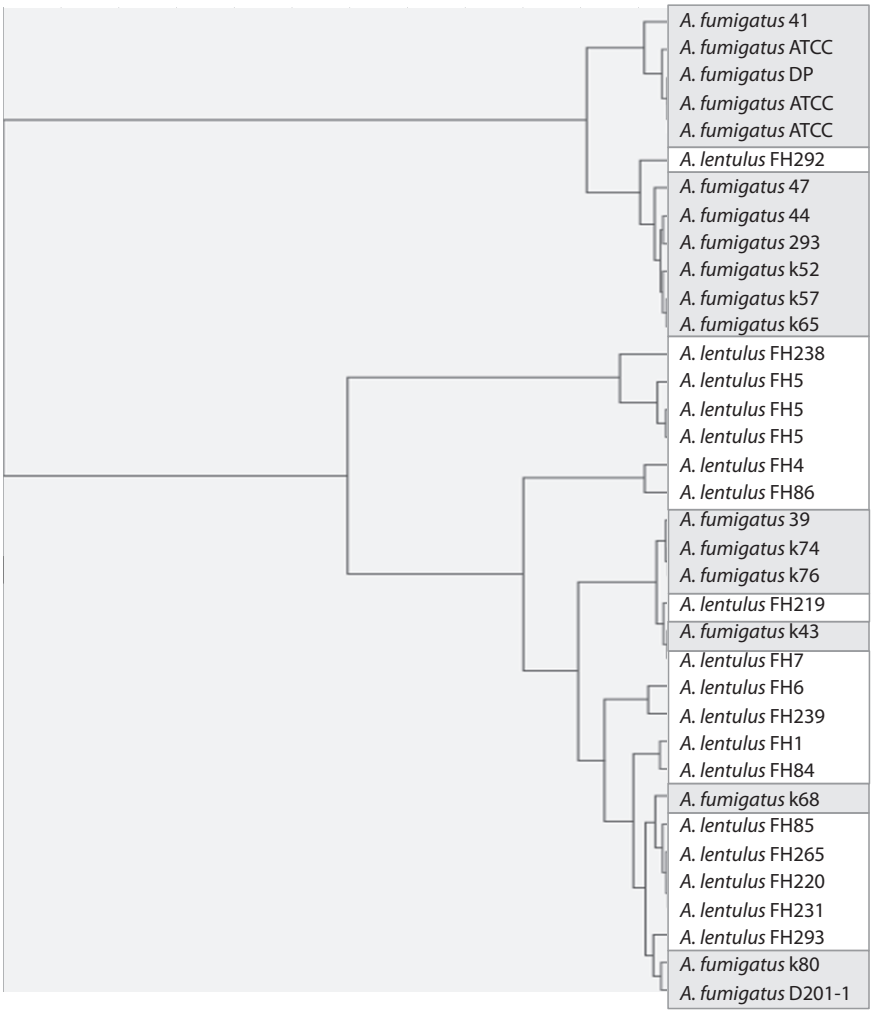

Figure 1. Clustering of 16 *Aspergillus fumigatus* and 16 *Aspergillus lentulus* strains, according to Spectracell BA Bacterial Analyzer.

cluster (Figure 1). Thus 10 out of15 *A. fumigatus* strains (67%) and 15 out of 16 of *A. lentulus* strains (94%) could be identified correctly. Overall, Raman spectroscopy performed on white conidia correctly classified 25 out of 32 (78%) of the fungal isolates included.

VITEK MS RUO distinguishes all *A. lentulus* isolates from *A. fumigatus*

For VITEK MS RUO, fungal strains were cultured on SDA for 48 h, according to standardized procedures. For one strain (*A. lentulus* FH292), no reproducible spectra were obtained owing to poor growth. This strain was thus excluded from analysis of VITEK MS RUO results. The spectra obtained were compared to the currently known spectra

for *Aspergillus* species in the bioMérieux database. Using these culture conditions, the median number of peaks detected, was 103 (range 80-140) for both species, which was well within the expected number of peaks of 100-150 obtained for other moulds with VITEK MS RUO. Analysis of mass spectra showed spectra that were closely related for both species, although subtle differences were observed (Figure 2). As can be seen in Figure 3, the 31 isolates fell into two separate clusters: one cluster with *Aspergillus lentulus* isolates only and one cluster with *Aspergillus fumigatus* isolates only. Two sequenced *Aspergillus flavus* and two sequenced *Aspergillus niger* isolates were included for comparison. These two species each fell into a separate cluster. Based on this technique, 16 out of 16 isolates of *A. fumigatus* (100%) and 15 out of 15 *A. lentulus* (100%) were correctly identified. Since it was previously reported that pigmented fungi yield spectra of poorer quality than less pigmented fungi when using VITEK MS RUO (14, 18), we also measured spectra of *A. fumigatus* and *A. lentulus* when cultured on SDA with 1% DMSO. The spectra did change slightly but the discriminatory power remained the same, again 16 out of 16 *A. fumigatus* and 15 out of 15 *A. lentulus* isolates were correctly identified (data not shown).

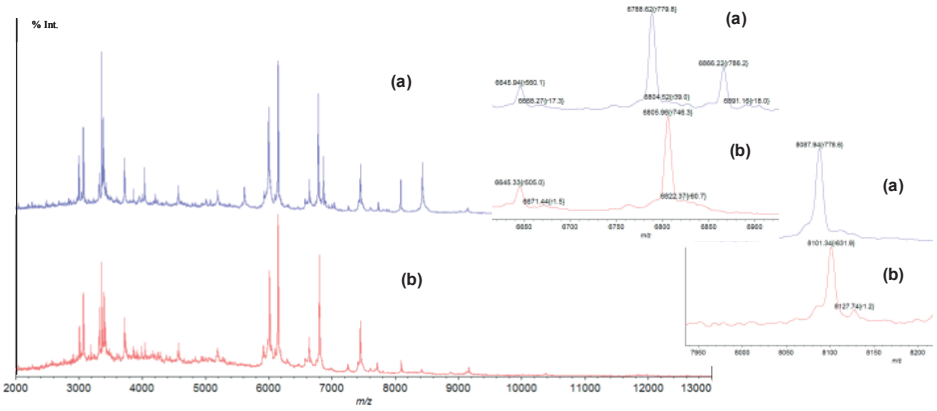

Figure 2. Results of VITEK MS identification of *Aspergillus lentulus* (A) and *Aspergillus fumigatus* (B).

VITEK MS RUO reduces the time needed to correctly identify *A. fumigatus* and *A. lentulus*

For diagnostic purposes, not only a correct identification but also the time frame in which this identification is obtained, is highly relevant. We therefore also investigated whether Raman spectroscopy and/or VITEK MS RUO could identify *A. fumigatus* and *A. lentulus* within a shorter time frame than PCR. For this comparison, the culture time was included for all techniques. The time frame for each step in the identification process is shown in Figure 4. PCR-based identification takes 57 h, while fungal identification by Raman spectroscopy took 100 h and identification by VITEK MS RUO only 50 h. The difference in

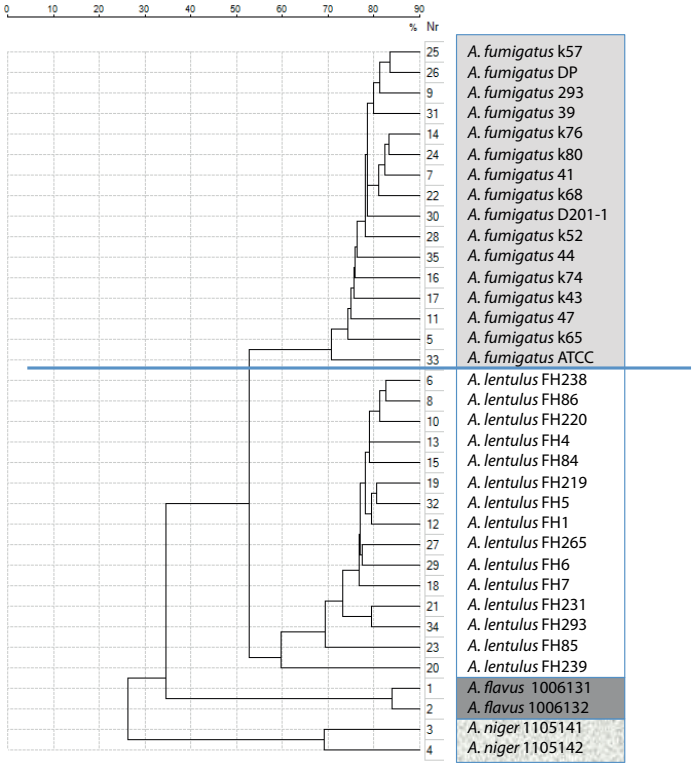

Figure 3. Clustering of 16 *A. fumigatus* and 15 *A. lentulus* isolates based on VITEK MS RUO data. 1 *A. lentulus* isolate was excluded from analysis due to poor growth.

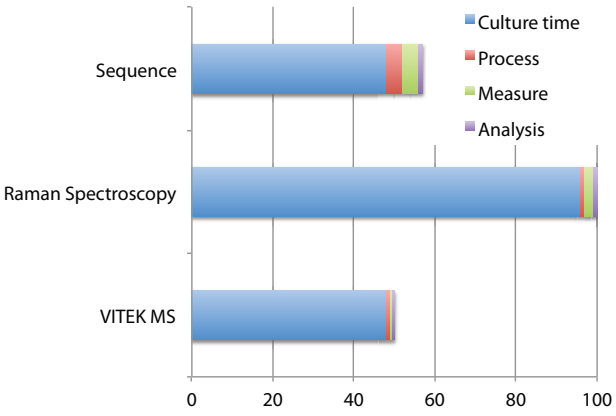

Figure 4. Timing identification of fungal strains using various methods.

time frame is mainly caused by the longer culture time needed for Raman spectroscopy. Conidia cannot be harvested before 96h of culture time, whereas 48h of culture time is sufficient to obtain suitable fungal material for analysis by VITEK MS RUO.

DISCUSSION

Both *Aspergillus lentulus* and *Aspergillus fumigatus* are able to cause invasive pulmonary infections in humans. However, since susceptibility to antifungal agents is not equal, it is important to correctly identify the causative fungal agent in order to optimize antifungal therapy. Since current techniques for identification are time consuming and not discriminative enough, we compared the performance of two newer identification techniques.

Raman spectroscopy was proven to be useful for the identification of bacteria (12, 13), though the identification of fungal species was not described previously. We showed here that Raman spectroscopy is an easy-to-operate technique, but a modification was needed to obtain reproducible spectra from pigmented fungi, since the expression of melanin caused interference. Culture medium modification resulted in reproducible spectra. Raman spectroscopy correctly classified 78% of *A. fumigatus* and *A. lentulus* isolates, but it is not considered discriminative enough to distinguish *A. lentulus* from *A. fumigatus*.

Regarding MALDI-TOF MS, it has also been reported that pigmented fungi yield spectra of poorer quality than less pigmented fungi (14, 18). We therefore determined whether or not modification of the culture medium, as applied to Raman spectroscopy samples, resulted in more reliable spectra. Under both culture conditions, 100% correct identifications of the isolates were obtained. Therefore, modification of the culture medium was not needed. Modification of the culture medium would add 48h of culture time. Besides, Bernardo *et al* and Arnold *et al* showed that MALDI-TOF MS spectra can vary with growth medium of the fungal isolate (19, 20); thus we conclude that it is not advisable to alter the growth medium.

In the past MALDI-TOF MS had already been described as a diagnostic tool in the identification of several *Aspergillus* species (14). Here we add *A. lentulus* to the list of species, which can be readily identified by MALDI-TOF MS. In our study both *A. fumigatus* and *A. lentulus* were identified with 100% accuracy. Additionally, identification of other species of the *Aspergillus fumigatus* species complex could be investigated, to test how specific MALDI-TOF MS can identify *A. lentulus*. Using the same technique, Hettick *et al* (14) were also able to identify *A. fumigatus* and Alanio *et al* (21) was able to identify 50 isolates with 96% accuracy. The 7 *A. lentulus* isolates included in Alanio's study were also

identified with 100% accuracy (21), on a different type of mass spectrometer (Bruker MALDI-TOF MS), but using similar methods. Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) was also able to distinguish between the protein profiles of *A. lentulus* (n=2) and *A. fumigatus* (n=2)(22), although it is more time-consuming. A more extensive study comparing SELDI-TOF MS with MALDI-TOF MS should be performed to determine which of these techniques is best suited as a diagnostic tool.

Other molecular techniques have been investigated recently as well. Araujo *et al* (23) and Serrano *et al* (24) identified *A. fumigatus* from several other fungal species, including *A. lentulus*, with a microsatellite-based PCR multiplex (23) and with a multiplex PCR based on β-tubulin and rodlet A partial gene sequences (24). Two and four *A. lentulus* strains respectively were included in the analysis. Furthermore Etienne *et al* (25) developed a microsphere-based Luminex assay to rapidly identify *A. fumigatus*. Non-*A. fumigatus* species were identified, though additional sequencing was required to identify the isolates. Using VITEK MS RUO, we correctly identified both *A. fumigatus* and *A. lentulus* in a single assay.

A. fumigatus and *A. lentulus* were proven the most prevalent species within the *Aspergillus fumigatus* complex (26). Though other species belonging to the section Fumigati, such as *N. ugadawae*, *N. pseudofisheri* and *A. viridinutans*, could be included in order to further investigate the discriminative power of MALDI-TOF MS for these species. Since these species were not readily available in the extended database of VITEK MS RUO spectra of bioMérieux, it was not possible to include these species in the current analysis.

For diagnostic purposes the time frame in which a correct identification is made is also relevant. Here we showed that fungal isolates could be identified within 100 h of the start of culture by Raman spectroscopy and within 50 h by VITEK MS RUO. This was less time-consuming than sequence-based identification, where culture is included. Direct diagnostics on patient material would be even less time-consuming, although sensitivity and specificity remain a problem (27). Since fungal spores are ubiquitous, many patients are colonized with *Aspergillus* species and discrimination between colonisation and infection could be problematic when diagnostics are performed directly on patient materials.

In conclusion, VITEK MS RUO is less time-consuming than Raman spectroscopy and more reliable. Therefore it suits better as a non-sequence based identification tool for the discrimination between *A. fumigatus* and *A. lentulus* than Raman spectroscopy. Since no adjustments to the culture medium were needed for proper identification, VITEK MS RUO is a suitable technique for identifying *A. lentulus* in daily practice. Further research to optimise the laboratory process may further shorten the diagnostic timeframe in which *A. lentulus* can be identified. Also, other species in the *Aspergillus fumigatus*

species complex could be tested. Still, both MALDI-TOF MS and Raman spectroscopy are dependent on a culture with a known low sensitivity (27). Identification techniques performed directly on clinical specimens could bypass fungal culture. If the causative infectious agent could be identified based on blood profiles or BAL profiles of patients, more time could be saved.

CONFLICT OF INTEREST

AB, VG and VM are employees of bioMérieux. None of them had any influence on the outcome of experiments or on the interpretation of results.

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CHAPTER 8

In vitro antifungal effect of branched histidine and lysine rich peptides (BHKPs) on *Aspergillus* species, *Candida* species and basidiomycetes

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Adapted from J Antimicrob Chemother 2011;66(7):1649-1652

ABSTRACT

The treatment of fungal infections in immunocompromised patients remains a challenge. Serious side effects and increasing resistance interfere with the success of antifungal therapy. Hence there is an ongoing need for new antifungal agents. A group of branched histidine and lysine-rich peptides (BHKPs) has been developed. These BHKPs have a mechanism of action similar to histatins. The aim of this study was to determine the antifungal spectrum of three BHKPs. Minimum inhibitory concentrations (MICs) were determined for 9 *Aspergillus* isolates, 5 *Candida* isolates, 21 *Cryptococcus* isolates and 8 *Trichosporon* isolates, according to the CLSI broth microdilution method. MICs were assessed in triplicate for the currently used antifungal agents amphotericin B, voriconazole, caspofungin, for the antimicrobial peptide histatin-5 (HST-5) and the BHKPs H2K4b, H3K4b(H) and H3K4B(G).

MICs of amphotericin B, voriconazole and caspofungin for the isolates tested were similar to MICs described in the literature. All isolates were resistant to HST-5 (MICs >16 mg/L). Both *Cryptococcus* and *Trichosporon* isolates seemed to be susceptible to the BHKPs, while *Aspergillus* species and *Candida* species were found to be resistant.

This study is the first to assess the antifungal activity of the BHKPs against a variety of fungal species. Of the three BHKPs tested, H2K4b showed highest antifungal potential. Studies in animal models of fungal infection are needed to determine the therapeutic activity of these peptides.

INTRODUCTION

The number of immunocompromised hosts is increasing over the last decades (1). As a result, opportunistic infections, among which both superficial and invasive fungal infections, frequently develop (2-4). These infections remain difficult to treat (3-7). As the use of a number of antifungal agents is limited due to serious side effects (8), new agents with different mechanisms of action have been developed. The currently used antifungal agents are divided into three major classes: the polyenes, among which amphotericin B (9); the azoles, of which voriconazole is frequently used (8, 9); and the echinocandins, with caspofungin as most important agent (10). In general, newly developed antifungal agents are derivatives of agents belonging to one of these classes. The use of newer azoles encounters problems with drug interactions and emerging resistance (5, 7). As a result there is an ongoing need for new antifungal agents.

A group of such novel antifungal agents are the branched histidine and lysine-rich peptides (BHKPs), originally developed as gene delivery agents (11). The new BHKPs that exhibit antifungal characteristics do not belong to any of the existing classes of antifungal agents (12). It is thought that the mechanism of action of the BHKPs is similar to that of histatins, which are a group of naturally occurring peptides with antimicrobial activity (12). These cationic peptides are found exclusively in mammalian saliva. Of the histatins, histatin-5 is considered the most potent antimicrobial peptide (13-15).

In a search for new antifungal agents, we aimed to determine the *in vitro* antifungal spectrum of three BHKPs. As reference agents we included three representatives of the currently used antifungal agents, being amphotericin B, voriconazole and caspofungin, and histatin-5 as another peptide. Antifungal susceptibility was determined by the broth microdilution method described in the CLSI guidelines (16, 17). In addition, the quantitative viability-based XTT assay was used (18).

MATERIALS & METHODS

Fungal strains

A broad panel of clinical isolates of fungi and yeasts, 9 *Aspergillus* strains, 5 *Candida* strains, 21 *Cryptococcus* strains and 8 *Trichosporon* species were included for susceptibility testing (Table 1). We included at least one reference strain for each species. The *Aspergillus* reference strains were *Aspergillus fumigatus* ATCC 204305, *Aspergillus niger* ATCC 16404, *Aspergillus terreus* CBS 594.65 and *Aspergillus flavus* CBS 625.66. The *Candida* reference strains were *Candida albicans* ATCC 90028 and *Candida glabrata* ATCC 66032.

Table 1. Median minimum inhibitory concentrations (MICs) in mg/L for 9 *Aspergillus* strains, 5 *Candida* strains, 21 *Cryptococcus* strains and 8 *Trichosporon* strains. Median values of 3 determinations are shown.

Strain		Antifungal agent							
		AMB	VRC	CAS	HST-5	H2K4b	H3K4b(H)	H3K4b(G)	MEC CAS†
A. fumigatus	ATCC 204305	0.5	1	128	>16	>1024	>1024	>1024	0.125
	EMC 1	0.5	0.5	128	>16	>1024	>1024	>1024	0.125
A. niger	ATCC 16404	0.125	1	64	>16	16	32	128	0.125
	21	0.125	1	128	>16	>1024	>1024	>1024	<0.063
	27	0.25	1	64	>16	16	256	512	<0.063
A. terreus	CBS 594.65	2	1	128	>16	>1024	>1024	>1024	<0.063
	11	0.25	1	128	>16	>1024	>1024	>1024	0.125
A. flavus	CBS 625.66	2	0.5	128	>16	>1024	>1024	>1024	<0.063
	25	1	1	128	>16	>1024	>1024	>1024	0.063
C. albicans	ATCC 90028	0.5	1	0.5	>16	>256	>256	>256	N/A
	W111	0.5	1	1	>16	>256	>256	>256	N/A
	W1016	0.5	4	1	>16	>256	>256	>256	N/A
C. glabrata	ATCC 66032	0.25	1	0.5	>16	>256	>256	>256	N/A
	1	0.5	0.5	0.063	>16	>256	>256	>256	N/A
C. neoformans	ATCC 90112	0.25	0.125	16	>16	8	64	128	N/A
var neoformans	CBS 131	0.125	0.125	16	>16	4	2	1	N/A
	CBS 889	0.016	0.016	16	>16	4	4	16	N/A
	B 29739	0.016	0.008	32	>16	32	64	64	N/A
	B 32287	0.031	0.125	16	>16	8	64	128	N/A
	B 34336	0.125	0.063	8	>16	8	16	8	N/A
	B 36203	0.008	0.016	8	>16	4	16	8	N/A
	B 36535	0.125	0.031	8	>16	4	4	2	N/A
C. neoformans	B32480	0.063	0.031	8	>16	4	16	8	N/A
var grubii	B 35735	0.031	0.031	16	>16	8	32	16	N/A
C. gattii	CBS 10090	0.25	0.5	32	>16	4	4	4	N/A
	CBS 10514	0.25	0.008	16	>16	2	4	4	N/A
	CBS 10865	0.5	0.008	16	>16	2	1	4	N/A
	CBS 11247	0.125	0.25	16	>16	4	4	4	N/A
	CBS 11542	0.125	1	16	>16	4	4	4	N/A
	CBS 11545	0.5	0.25	16	>16	2	4	4	N/A
	CBS 11752	0.5	0.125	32	>16	4	4	8	N/A
	CBS 11807	0.125	0.5	16	>16	2	2	4	N/A
	CBS11816	0.125	1	16	>16	4	4	8	N/A
	A 47496	0.063	0.008	32	>16	4	4	8	N/A
	ICB 107	2	1	128	>16	4	2	2	N/A
T. asahii	CBS 2479	1	0.25	32	>16	4	4	128	N/A
T. inkin	CBS 5585	0.25	0.063	16	>16	4	8	128	N/A

Table 1. Median minimum inhibitory concentrations (MICs) in mg/L for 9 *Aspergillus* strains, 5 *Candida* strains, 21 *Cryptococcus* strains and 8 *Trichosporon* strains. Median values of 3 determinations are shown. (continued)

Strain		Antifungal agent							
		AMB	VRC	CAS	HST-5	H2K4b	H3K4b(H)	H3K4b(G)	MEC CAS†
T. mucoides	CBS 6939	0.063	0.5	16	>16	8	16	64	N/A
	CBS 7616	0.063	0.125	32	>16	32	64	128	N/A
	CBS 7625	0.063	0.25	16	>16	8	8	128	N/A
	CBS 7626	0.016	0.008	8	>16	16	16	32	N/A
	CBS 7653	0.016	0.008	8	>16	16	16	16	N/A
	CBS 7722	0.063	0.125	16	>16	8	32	128	N/A

AMB, amphotericin B; VRC, voriconazole; CAS, caspofungin; HST-5, histatin-5. H2K4b and H3K4b(H) and H3K4B(G), BHKPs. † MEC CAS Minimum effective concentration for Caspofungin. N/A not applicable.

The *Cryptococcus* reference strains were *Cryptococcus neoformans* ATCC 90112 and *Cryptococcus gattii* CBS 10090. The *Trichosporon mucoides* reference strain was *Trichosporon mucoides* CBS 6939. The other *Trichosporon* species tested were *Trichosporon asahii* CBS 2479 and *T. inkin* CBS 5585.

Antifungal agents

Amphotericin B (Bristol-Myers Squibb, Woerden, the Netherlands), caspofungin (Merck and Company, Rahway, NJ, USA) and voriconazole (Pfizer BV, Capelle a/d IJssel, the Netherlands) were dissolved as indicated in the CLSI guidelines and stored at –80°C until use. Histatin-5 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and H2K4b, H3K4b(H) and H3K4B(G) (Figure 1, Aparna BioSciences, Washington, USA) were dissolved in sterile water and stored at –80°C until use. Twofold serial dilutions were prepared. Ampho-

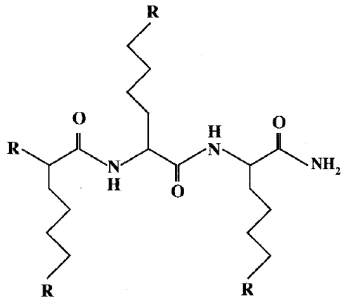

Figure 1. Schematic structure of branched Histidine and lysine rich peptides (BHKPs). The peptides consist of terminal branches, originating from a lysine core. This lysine core consists of three lysines. R represents KHKHHKHHKHHKHHKHHKHK for H2K4b, KHHHKHHHKHHHHKHHHK for H3K4b(H) and KHHHKHHHKHHGH-KHHHK for H3K4b(G).

tericin B, voriconazole and histatin-5 were tested in the concentration range of 0.008-16 mg/L, caspofungin in the concentration range of 0.063-128 mg/L and the BHKPs in the concentration range of 0.5 – 128 mg/L.

In vitro antifungal susceptibility testing by broth microdilution method

All isolates were tested according to the broth microdilution methods for qualitative susceptibility testing of filamentous fungi and yeasts, respectively, described by the Clinical and Laboratory Standards Institute (16, 17). In short, for preparation of the inocula, fungi were cultured on Sabouraud's dextrose agar (Oxoid Ltd, Basingstoke, England), conidia from filamentous fungi were harvested and washed in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). A standardized inoculum was prepared in RPMI-1640 (with L-glutamine but without bicarbonate) (Lonza, Verviers, Belgium), buffered to pH 7.0 with 0.165 M 3-*N*-morpholinopropanesulphonic acid (MOPS) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). NaCl 3g/L was added to the RPMI to simulate physiological salt concentrations of human blood. To determine the antifungal activity of the BHKPs in the presence of serum, 5% fetal calf serum (not heat-inactivated, Gibco, Breda, the Netherlands) was added. The inoculum was standardized with a haemocytometer (Bürker-Türk, Marienfeld, Germany) to a final concentration containing 2.3×10^5 conidia/ml. For yeasts, colonies were suspended in sterile saline and adjusted to 0.5 McFarland, with a final inoculum of 1.5×10^3 CFU/ml.

MICs were determined in triplicate and read visually according to the CLSI guidelines. In short, MICs were defined as the lowest concentration of antifungal agent, which prevented visible growth of the fungus or yeast. For yeasts, the MIC for voriconazole and caspofungin was defined as the lowest concentration in which a prominent decrease in turbidity is observed. Minimum effective concentrations (MECs) for caspofungin were determined for all *Aspergillus* strains. The MEC was defined as the lowest concentration of caspofungin causing abnormal growth with broad-based, short branched hyphae (19, 20). MECs also were read visually.

In vitro antifungal activity by viability-based XTT assay

The viable fungal mass was assessed in a quantitative assay, as described earlier (18). In this assay, the metabolic activity of the fungal mass is quantified in terms of mitochondrial dehydrogenase activity converting XTT (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) into formazan. The formazan excreted into the medium after 2 hours of incubation was determined spectrophotometrically at 450 nm. The percentage of viable fungal mass was calculated. We used this method to compare visually read MICs (CLSI method) to calculated MICs (XTT assay).

Statistical analysis

Differences in MICs were analyzed by two-tailed Mann-Whitney test (GraphPad Prism Software, San Diego, USA).

RESULTS

Table 1 shows the *in vitro* susceptibilities of 9 *Aspergillus* isolates, 5 *Candida* isolates, 21 *Cryptococcus* isolates and 8 *Trichosporon* isolates. All the isolates tested were found to be susceptible to amphotericin B and voriconazole.

Aspergillus species and *Candida* species

For *Aspergillus* species, MICs for caspofungin were high, with a median MIC of 128 mg/L. However, the MECs were much lower (median 0.063 mg/L). All *Candida* species tested were inhibited by ≤ 1 mg/L caspofungin (median 0.5 mg/L). Table 1 also shows that *Aspergillus* species and *Candida* species were not susceptible to histatin-5 (MICs >16 mg/L) or to the three new peptides H2K4b, H3K4b(H) and H3K4B(G) (MICs >128 mg/L), with the exception of *A. niger*. The median MICs for *A. niger* were 16 mg/L for H2K4b and >128 mg/L for H3K4b(H) and H3K4B(G). This fungus was apparently susceptible to H2K4b only. Since caspofungin induces morphological changes at concentrations below MIC, we also determined the morphology of BHKP-treated fungi. Microscopic observations show that exposure to H2K4b tested at the concentration of 128 mg/L, which is below MIC, did not result in morphological changes of hyphae in *A. fumigatus* ATCC 204305 (data not shown).

Cryptococcus species and *Trichosporon* species

Unlike *Aspergillus* and *Candida* species, *Cryptococcus* species were more susceptible to the BHKPs. The median MICs for *Cryptococcus* species were 4, 4 and 8 mg/L for the three BHKPs, respectively. *C. gattii* species were inhibited by BHKPs at a lower concentration than *C. neoformans* species For H2K4b, the median MICs were 8 mg/L for *C. neoformans* species and 4 mg/L for *C. gattii* species ($p = 0.003$). For H3K4b(H), the median MICs were 16 mg/L for *C. neoformans* species and 4 mg/L for *C. gattii* species ($p = 0.004$). For H3K4B(G), the median MICs were 16 mg/L for *C. neoformans* species and 4 mg/L for *C. gattii* species ($p = 0.035$).

Based on the above, we noted that H2K4b is more potent in the inhibition of growth of *Cryptococcus* species when compared to the other BHKPs. The presence of a capsule (microscopic observation using Indian ink) did not influence susceptibility. Neither was the susceptibility of *Cryptococcus* species changed by the addition of 5% fetal calf serum (not heat-inactivated) (data not shown).

Since *Cryptococcus* species were susceptible to the BHKPs, we wondered whether this phenomenon was also found in other pathogenic basidiomycetous species. We therefore determined MICs for 8 *Trichosporon* strains. The median MICs were 8 mg/L for H2K4b, 16 mg/L for H3K4b(H) and 128 mg/L H3K4B(G), respectively. *Cryptococcus* species and *Trichosporon* species were resistant to histatin-5. The median MICs were >16 mg/L for all species tested. *Cryptococcus* species and *Trichosporon* species were both inhibited by the three BHKPs, though apparently H2K4b resulted in the most effective inhibition.

In vitro antifungal activity assessed by viability-based XTT assay

In addition to the broth microdilution methods, we also investigated the viable fungal mass using the XTT assay. Increasing concentrations of voriconazole for example induced a decrease in viable fungal mass, as observed by decreased concentration of colored formazan (data not shown). For H2K4b, a concentration-dependent effect was not observed when using the broth microdilution method. However, in the viability assay, we noticed a peculiar phenomenon after exposure to high concentrations of the BHKPs (>64 mg/L). XTT was converted into formazan, though the formazan was not excreted. Due to an unknown change in the fungal cells, the XTT became trapped in the cell and the fungal mass remained dark red. Since calculating the viable fungal mass is based on measuring optical density and hence on the excretion of formazan, the percentage found did not represent the true percentage of viable fungal mass. This discrepancy between visually read MICs and calculated MICs by viability staining was similar for the three BHKPs and all *Aspergillus* species investigated.

DISCUSSION

In this study, we explored the antifungal spectrum of three novel, potentially antifungal, branched histidine and lysine-rich peptides. MICs for BHKPs were compared to MICs for currently used antifungal agents. MICs obtained for amphotericin B, voriconazole and caspofngin were consistent with those described in the literature (16, 17, 21). The MEC values we found for caspofungin were much lower than the MIC values, which is also consistent with literature (16, 20). We showed that *Cryptococcus* species and *Trichosporon* species were susceptible to the BHKPs H2K4b, H3K4b(H) and H3K4B(G), whereas *Aspergillus* species and *Candida* species were not. H2K4b seemed to be superior in *Cryptococcus* species and in *Trichosporon* species MIC values for H2K4b were lower than for H3K4b(H) and H3K4B(G).

We found a difference in susceptibility towards the BHKPs for *Aspergillus* species and *Candida* species, compared to *Cryptococcus* species and *Trichosporon* species. A possible explanation for this difference in susceptibility could be that *Cryptococcus* species and *Trichosporon* species differ from *Aspergillus* species and *Candida* species in phylogenetic affiliation (21, 22). The genera *Cryptococcus* and *Trichosporon* belong to the Basidiomycota, whereas both *Aspergillus* and *Candida* belong to the Ascomycota (21, 22). Basidiomycota and Ascomycota differ with respect to their cell wall composition. For instance, *Cryptococcus* species and *Trichosporon* species both express glucuronoxylomannan on their cell wall, whereas *Aspergillus* species and *Candida* species lack glucuronoxylomannan (23).

Zhu *et al* presumed that the mechanism of action of BHKPs is similar to the mechanism of action of histatin-5, since it is also a histidine-rich peptide (12). In *Candida* species histatin-5 binds to the Ssa1/2 surface protein (24), like other antifungal peptides, such as the human β-defensins 2 and 3 (25). The difference in susceptibility could also be the result of genetic differences in the Ssa1/2 receptor.

After binding the Ssa1/2 receptor, histatin-5 becomes internalized, where it opens the ion channels and causes dysfunction of the Trk-1p transporter (12). This transporter is the main target in cysteine-free peptides, like human neutrophil defensin 1 (25), lactoferricin (25) and bactenecins (26) and is involved in their fungicidal activity. However, the Trk-1p transporter is required more explicitly in the antifungal activity of histatin-5 than of the other peptides mentioned (25). Perhaps the Trk-1p transporter is more essential in fungicidal activity for the BHKPs in *Cryptococcus* species and *Trichosporon* species than in *Aspergillus* species and *Candida* species, resulting in increased resistance for the latter species.

In *C. albicans*, the effects of Trk-1p transporter dysfunction are efflux of ATP and other small nucleotides and, finally, cell death (12). In *Aspergillus*, this mechanism might be different. When we performed the viability staining using XTT, XTT became trapped inside the *Aspergillus* cells. Internalization of the BHKPs possibly causes another type of dysfunction of the Trk-1p transporter in *Aspergillus* species than in *Candida* species. The function of the transporter might become blocked by BHKPs, and thus the influx of potassium and the efflux of chloride are inhibited. Other ions would accumulate in the cell as well. This explains the observed accumulation of formazan in the mycelium when performing the XTT assay.

From our results, we noted that growth of *Cryptococcus* species and *Trichosporon* species was inhibited at relatively low concentrations of all BHKPs tested. Therefore the BHKPs might be useful as a new class of antifungal agents for these fungal diseases, for which mortality and morbidity remain substantial (27). Infections with *Trichosporon* species are relatively rare, while infections with *Cryptococcus* species are more common. (28)

Trichosporon species cause both superficial and deep-seated infections, the latter mainly occurring in the immunocompromised patient (29). Infections due to these invasive infections are potentially life-threatening (30, 31) Treatment with triazoles has shown to improve survival of patients with trichosporonosis, though mortality remains substantial (29, 30, 32) *Cryptococcus* species are the most common aetiological agents of fungal meningitis, occurring mainly in the immunocompromised host, though not exclusively (33-35). *C. neoformans* causes infections in immunocompromised patients, whereas *C. gattii* most commonly infects otherwise healthy patients (35-37). The spectrum of cryptococcal disease ranges from asymptomatic to invasive meningo-encephalitis (35, 36). Even though these species are susceptible towards the existing antifungal agents (38), cryptococcosis remains to result in high mortality, especially in children (35, 39, 40). Most antifungal agents do not cross the blood-brain barrier, and are not able to reach the site of infection (41). For BHKPs, the capacity to cross the blood-brain barrier is not known and might be studied in an animal model of experimental cryptococcosis.

The antifungal activity of the BHKPs was proven, though tolerability and cytotoxicity should also be investigated. With respect to tolerability, Zhu *et al* (12) reported that a concentration of 100 mg/L of H2K4b caused less than 1% cell death in human cell lines (12). Leng *et al* histologically observed no toxic side effects in various organs of mice after intravascular injection of H2K4b (100 µg five times, every three days)(42). These data on potential toxic side effects combined with our data on antifungal activity, suggest that BHKPs could be a new class of antifungal agents with little side effects.

In conclusion, the present study shows that the BHKPs have high antifungal activity against the basidiomycetes *Cryptococcus* species and *Trichosporon* species and may open new ways to reduce morbidity and mortality. Further studies are needed to explore the potential activity of these peptides in therapy of these difficult-to-treat infections.

ACKNOWLEDGEMENTS

The authors thank prof. dr.dr. A. van Belkum for contributing in this research.

CONFLICT OF INTEREST

This work was partly supported by Aparna Biosciences Corp. MW is an employee of Aparna Biosciences Corp and declares he has competing financial interest including ownership of the company. He had no influence on the outcome of experiments or on the interpretation of results.

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CHAPTER 9

Summarizing discussion

Invasive pulmonary aspergillosis (IPA) remains an infection with high morbidity and mortality in immunocompromised patients. It is most often caused by *Aspergillus fumigatus* (1) and both diagnosis and treatment of IPA can be challenging. The echinocandin caspofungin is one of the currently used antifungal agents. Interestingly, *in vitro* the fungistatic action of caspofungin is evident only at high concentrations (2). However, the therapeutic results of treatment with caspofungin are more favorable. In this thesis we hypothesized that the cell wall composition of echinocandin susceptible *A. fumigatus* is altered by caspofungin. The altered fungal cell walls subsequently stimulate several components of the immune system resulting in a better clinical outcome. The data presented in this thesis document the modifying influence of caspofungin on the fungal cell wall composition, and its subsequent effect on the immune response of the host.

CASPOFUNGIN: EFFECTS ON THE COMPOSITION OF THE FUNGAL CELL WALL

The mechanism of action of the echinocandins is based on the inhibition of synthesis of β-glucan, one of two major constituents of the fungal cell wall (50-60% of the cell wall by dry weight)(3). The other major polysaccharide of the fungal cell wall is chitin (10-20%) (3). Together with galactomannan these polysaccharides form a solid cell wall, providing turgor, which will prevent the fungal cell from collapsing. Furthermore, this rigid cell wall also protects the fungus against the immune system of the host. Caspofungin inhibits the synthesis of β-glucan in the cell wall of fungi and yeasts (4-6). As shown in **Chapter 2**, exposure of *A. fumigatus* to caspofungin resulted in a concentration-dependent decrease of β-glucan in the fungal cell wall to 80%. It was therefore envisioned that *A. fumigatus* would respond to caspofungin challenge by the upregulation of other cell wall components so as to maintain its strength and to keep turgor.

Indeed, in caspofungin-exposed *A. fumigatus*, the chitin component of the cell wall proved to be upregulated, i.e. constituted a greater part of the cell wall mass. Exposure to chitin synthesis inhibitor nikkomycin Z resulted in a decrease of the chitin component of the fungal cell wall, but did not affect its β-glucan content, which remained similar. Also in other fungi including other *Aspergillus* species, *Candida* species and *Saccharomyces* species increased chitin levels were detected after their exposure to caspofungin (4, 7-14). Combination of caspofungin and nikkomycin Z led to a crippled cell wall and the fungus was no longer able to maintain a rigid cell wall. This resulted in dramatic morphologic changes, even at low drug concentrations, as demonstrated in **Chapter 2** and by others (7, 15, 16). Furthermore, this phenomenon was not only found when nikkomycin Z was combined with caspofungin, but also in combinations with other echinocandins such as anidulafungin (7) or micafungin (16). These *in vitro* results have been translated

into *in vivo* studies in experimental infections. Clemons *et al* showed that nikkomycin Z combined with micafungin led to prolonged survival in a murine model of pulmonary aspergillosis (17). Furthermore, in the samen infection model, Luque *et al* reported that combination treatment resulted in increased mouse survival rates from 20-30% after single agent treatment, and to 100% survival of mice when treated with combination therapy (18).

Although these *in vitro* and *in vivo* results look promising, there is currently no clinical data on this combination treatment in patients with IPA. This can partly be explained by the fact that nikkomycin Z is currently not freely available. The drug was developed in the 1970s, however clinical development of nikkomycin Z was not successful. In the early 2000's, the University of Arizona invested in clinical research of nikkomycin Z in the treatment of Valley fever (coccidioidomycosis; https://www.vfce.arizona.edu/resources/pdf/bio5_summary_nikz_ development_plan.pdf).

Since combining nikkomycin Z with echinocandins was not possible in patients, other means have been tried *in vitro* and *in vivo* to inhibit chitin synthesis, combined with inhibition of β-glucan synthesis by echinocandins. Fortwendel and others demonstrated that the increase of chitin synthesis in response to caspofungin treatment was caused by a universal stress response pathway, mediated by calcineurin (8, 9, 11, 19-21). As a result, inhibiting the calcineurin mediated stress response also led to inhibition of the increase in chitin biosynthesis in reaction to caspofungin treatment. Based on these mechanisms, it is not surprising that the combination of caspofungin (blocking β-glucan synthesis) and calcineurin inhibitors (blocking the stress response leading to chitin increase), results in synergy between these agents (8, 9, 14, 19, 22). So in the future we might be able to enhance the therapeutic efficacy of caspofungin by inhibiting the increase in chitin synthesis through either direct inhibition of the chitin synthesis pathway with nikkomycin Z, or indirect inhibition using calcineurin inhibitors. Although in stdudies using an *A. fumigatus* strain with mutated proteins which resulted in impaired activity of the calcineurin pathway, synergy of nikkomycin Z and caspofungin *in vitro* was not confirmed (9).

As became clear from the above, inhibition of chitin synthesis is important to enhance the antifungal effect of echinocandins. It has been found that there is even a correlation between the amount of chitin in the fungal cell wall and the susceptibility of the fungus towards caspofungin (23). In fungi and yeasts with naturally high levels of chitin in their cell wall, the antifungal effect of caspofungin is probably less. Therefore, a high chitin content in the fungus provides a mechanism for the fungus to escape from the fungistatic effect of caspofungin. In the treatment of these fungal isolates, combination therapy with chitin synthesis inhibitors and β-glucan synthesis inhibitors could result in

better killing of these relatively caspofungin-resistant fungal species. On the other hand, Lee *et al* observed that *Candida* species with increased chitin levels are less virulent in a murine model than species with normal chitin levels (24). An *A. fumigatus* isolate with increased chitin was also found to be less virulent compared to other isolates (25). Since treatment with caspofungin results in an increased chitin level in the fungal cell wall of *A. fumigatus*, this could result in decreased virulence, thus improving clinical outcome. We conclude that the mechanism(s) through which chitin exactly plays a role in IPA development remains to be fully elucidated.

CYTOKINE RESPONSE IN RATS WITH IPA

Back to the mechanism of action of caspofungin, our first thought was that the altered ratios in chitin and β-glucan in the fungal cell wall could influence the immune system of the host. Both chitin and β-glucan are considered pathogen-associated molecular patterns (PAMPs) in the fungal cell wall, which act as signaling molecules on the outside of pathogens. PAMPs are recognized by pattern recognition receptors (PRRs), which are localized on immune cells of the host. Subsequent to attachment, an immune reaction is triggered through activation of a specific pathway, mounting a pro- or anti-inflammatory response. Each specific PAMP binds to a specific PRR and causes a certain response. The PAMP β-glucan is recognized by Dectin-1 and complement receptor 3 (CR3), which are supported by toll-like receptor (TLR)2 (26, 27). For the PAMP chitin no specific receptor was identified, however chitin, or chitin particles, can cause co-stimulation of TLR2, which leads to activation of many inflammatory pathways (28-34). Not only TLR2, but also TLR4 was demonstrated to be important in the recognition of *A. fumigatus* (35).

In order to answer the question whether an altered composition of the fungal cell wall would result in an altered immune response in the host, we first had to determine the normal immune response towards IPA in our rat model. Many studies investigating cytokines in aspergillosis were published. Most studies focused on only a small panel of cytokines or used models of immunocompetent hosts. In **Chapter 3** we describe the cytokine response in neutropenic rats with IPA. We determined levels of 24 pro- and anti-inflammatory cytokines as well as chemokines and regulatory cytokines in 4 groups of rats. Cytokine responses in immunocompetent rats, inoculated with *A. fumigatus* conidia were compared to cytokine responses in immunocompromised rats with IPA. First, we observed that the dynamics in the levels of local cytokines and chemokines at the site of infection, i.e. in the lungs, differed from that of the cytokines circulating in the bloodstream. In the lungs, IPA clearly induced pro-inflammatory interleukin (IL)-1α and IL-6. Furthermore, the chemokines interferon-gamma induced protein (IP)-10, keratinocyte chemoattractant (KC, the murine functional homologue of human CXCL-8), monocyte

chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α were all significantly increased rats with IPA. However, the levels of anti-inflammatory cytokines in the lungs did not change during IPA. In contrast, in serum we found increased levels of pro-inflammatory granulocyte macrophage-stimulating factor (GM-CSF) and IL-12p70 and of anti-inflammatory IL-5 and IL-10. Chemokines IP-10 and KC were also found significantly increased in serum of rats with IPA. Many investigators determined cytokine responses in mammals exposed to *A. fumigatus*, however none studied such a broad panel of cytokines and chemokines. Tomee *et al* (36) stimulated human airway epithelial cells with *A. fumigatus* and found similar results for production of IL-6, IL-8 and MCP-1, but found no increase in GM-CSF, which we found exclusively in the bloodstream. Stimulation of dectin-1 was previously shown to induce IL-6 and IL-10 (37) and also in patients IL-10 was found increased after infection with *A. fumigatus* (28, 38). It is clear that some evidence is conflicting. Activation of dectin-1 is known to stimulate IL-17 and TNF-α (31, 37) and likewise chitin is known to stimulate IL-17 and IL-12/23p40 but not IL-12p70 (39). Our results are not in line with these earlier observations by others. However, differences in route of infection, *in vivo* or *ex vivo* study design and the selection of host species may influence results and, therefore, the observed differences in cytokine responses may, in part, help explain the results.

CASPOFUNGIN: EFFECTS OF CHANGED PAMPS ON THE CYTOKINE RESPONSE

Changes in the fungal cell wall as a result of exposure to caspofungin, cause an alteration in the relative contribution of the PAMPs chitin and β-glucan. This change in cell wall composition is likely to lead to modifications in the activation of PAMP-TLR molecular pathways and thus in an altered immune response in the host.

In **Chapter 3** we investigated the cytokine response in caspofungin-treated rats with IPA. Treatment with caspofungin resulted in 100% rat survival as opposed to 100% rat mortality among untreated rats. In general we observed that the cytokine responses in caspofungin-treated rats was very similar to that in untreated rats. Some subtle differences occurred, though no striking differences were found. However, these seemingly subtle differences might be responsible for the good clinical outcome after treatment with caspofungin.

We found that in the lungs, caspofungin caused an attenuation of the increase in pro-inflammatory IL-1α and IL-6 and the chemokines IP-10, KC, MCP-1 and MIP-1α. Furthermore the increase in systemic inflammatory GM-CSF and IL-12p70, as well as the anti-inflammatory IL-5 and IL-10 and chemokines IP-10 and KC was attenuated by treatment with caspofungin. In summary, caspofungin limited the increase in several locally and systemically produced cytokines and chemokines, which coincided with

100% survival of the rats. Apart from limiting the β-glucan synthesis, caspofungin also unmasks the deeper cell wall layers and thus increases the exposure of the β-glucan to the immune system of the host (8, 10, 24, 40-42). These immunomodulatory effects of caspofungin have been investigated previously. There is conflicting evidence on the effects of the increased exposure to β-glucan. An increased pro-inflammatory response in reaction to *Aspergillus* species and in *Candida* species was described (40-42), but also a decreased inflammatory response was reported after treatment with micafungin (43), which is more in line with our results (**Chapter 3**). Olson *et al* investigated the cytokine response *in vivo* in neutropenic mice with *Aspergillus flavus* infection (44) and cytokines in caspofungin-treated rats were compared to those in untreated rats. They found an increased mortality in mice treated with caspofungin due to increased levels of pro-inflammatory cytokines, including TNF-α. Increased levels of pro-inflammatory cytokines seem to coincide with decreased survival, whether or not as a result of treatment with antifungal agents.

Finally, Netea *et al* described that balance between TLR2 and TLR4 seems to be crucial for the outcome of fungal infections (31). As a result of treatment with caspofungin, PAMPs are changed and the TLR2/TLR4 balance could be shifted, resulting in favorable outcome.

In conclusion, our results are in line with the previously reported hypothesis that an excessive immune response is responsible for tissue injury and is associated with mortality (45).

CHITINASE PRODUCTION IN RATS WITH IPA

Based upon the cytokine response, we could not completely explain the clinical results after treatment with caspofungin. Therefore, we continued by investigating another component of the immune system: the chitinase response.

In **Chapter 2** we showed that treatment with caspofungin led to upregulated chitin contents of the fungal cell wall. When a mammalian host encounters chitin polymers, it can produce chitinases, which are chitin-cleaving enzymes. Two types of chitinases have been discovered in humans so far: chitotriosidase and acidic mammalian chitinase (AMCase)(46). Since fungi contain chitin, it may be expected that mammals experiencing invasive fungal infection such as IPA, produce increased levels of chitinases.

In **Chapter 4** we investigated the role of chitinases in the host with IPA. In a rat model, chitotriosidase and AMCase production was investigated after infection with *A. fumigatus*. We investigated both immunocompetent and immunocompromised hosts and measured serum and pulmonary chitinase activity. We showed that in immunocompromised

rats the production of both chitotriosidase and AMCase are significantly increased in IPA and that it is increasing with progressing infection, parallel with increasing fungal load. In immunocompetent rats however, both chitinases are only slightly and transiently increased after challenge with *A. fumigatus* conidia. After clearance of the conidia the chitinase production declines again to undetectable levels. Furthermore, we showed that serum chitinase and tissue chitinase kinetics do not parallel each other, and that chitotriosidase and AMCase are both found in the lung at the site of infection.

CASPOFUNGIN: EFFECTS ON CHITINASE PRODUCTION

Infection with fungal pathogens containing more chitin (for example as a result of treatment with caspofungin) could lead to increased levels of chitinases produced by the infected host (47, 48). These chitinases lyse the fungal cell wall, which could assist in clearance of the fungus. These indirect effects of the antifungal agent may help explain the favorable clinical outcome of treatment with caspofungin whilst the *in vitro* effects of caspofungin on the fungus are poor.

We therefore hypothesized that chitinase levels in caspofungin-treated rats with IPA are higher than in untreated rats with IPA, as result of the chitin upregulation of the fungal cell wall. However, partly due to small numbers of rats in the experiments, a significant difference could not be detected between caspofungin-treated (surviving) rats and the untreated (non-surviving) rats (**Chapter 4**). Interestingly and in contrast to our expectation, there was a trend towards lower chitinase levels in caspofungin-treated rats. This was probably due to the overriding influence of the fungal load on the host's chitinase response, fungal loads being much lower in caspofungin-treated surviving rats compared to the increasing fungal loads in rats left untreated and dying from IPA. When the overall fungal load remains lower, the overall amount of chitin is probably also lower, resulting in a lower production of chitinases by the infected host. However, we concluded that chitinases do play a significant role in the host's response to IPA, though other factors are also involved.

The effects of increased levels of chitinase have not been established by others. Increased levels of chitotriosidase were reported to present in the lung fluid and in lung macrophages of patients suffering from chronic obstructive lung disease, especially among smokers (49). The same authors also showed that chitotriosidase was able to stimulate the production and release of IL-8 and MCP-1 from alveolar macrophages in humans, even more from macrophages from smokers and COPD patients compared to healthy controls (49) However, the role of these chitinases in IPA remains to be elucidated. We showed that when *A. fumigatus* is treated with caspofungin, a combination

of chitotriosidase and AMCase can lyse the fungal cell wall, whereas untreated *A. fumigatus* seems to be unaffected by the combination of the two chitinases. Caspofungin, therefore, somehow leads to enhanced fungal susceptibility to the combination of these chitinases.

Caspofungin is known to unmask the deeper cell wall layers (8, 10, 24, 40-42) containing the chitin-β-glucan matrix. When the deeper cell wall becomes more accessible to lysing enzymes such as chitinases, chitin-degrading effects would be expected to occur. When chitinase levels are high and when chitinases can better reach the chitin, large chitin polymers can be cleaved into smaller particles. Previously it was reported that the size of chitin particles determines the immune response of the host (32, 39, 50). Da Silva *et al* investigated the immune response mounted by different sizes of chitin particles (32). They found that large chitin particles (70-100 μm) and very small chitin particles (<2 μm, created by cleaving of larger particles due to chitinase) are immunologically inert, while somewhat larger but still small chitin particles (<40 μm but mostly 2-10 μm) as well as intermediate chitin particles (40-70 μm) each mount a specific immune response. For example, small chitin particles induce the release of the anti-inflammatory IL-10 and the pro-inflammatory tumor necrosis factor (TNF), whereas intermediate chitin particles only induce TNF (32). Also, intermediate particles induced IL-17, whereas larger chitin particles did not (39). Furthermore Shibata *et al* showed that chitin particles of 1-10 μm induce IFN-γ via IL-12 and TNF-α production, whereas larger particles of 50-100 μm did not induce IL-12 or IFN-γ (50). The exact size of the chitin polymers or particles used in our challenge experiments described in **Chapter 3** is not known. Since in our rat model *A. fumigatus* conidia are used to induce the infection, which after inoculation subsequently develop into hyphae which do not present chitin particles of a well defined size range, the immune response reported in **Chapter 3** was rather induced by the combination of chitin, β-glucan and mannan present in the cell walls of the conidia and hyphae. This could explain why our results, although directly observed in an infection model, do not completely match the hypothesized cytokine response for isolated chitin particles.

When large chitin polymers are cleaved into smaller particles to a significant degree, the immune response probably changes. Smaller chitin particles skew the immune response towards Th1 instead of the Th2 response that is mounted by large chitin polymers (51).

Strong *et al* confirmed that application of chitin microparticles (mostly <1 μm) increased levels of Th1 cytokines IL-12, IFN-γ and TNF-α, finally resulting in a decrease of lung inflammation (52). Again our results in **Chapter 3** do not completely match this cytokine profile, probably due to the difference in immune response to chitin particles versus live *A. fumigatus*.

Application of small chitin particles (1-10 μm) in an allergic animal model down-regulated Th2 cytokines as well as IgE production and lung eosinophilia (53). In a fulminant

infection, the inflammatory response could lead to increased inflammatory damage of tissue when the cytokine response is not down-regulated. Hence the host benefits from smaller chitin particles that induce anti-inflammatory cytokines. These cytokines limit the inflammatory reaction and terminate the immune reaction in order to prevent tissue damage (32). Degradation of chitin by chitinases thus ultimately results in a reduced immune response (54). In summary, increased levels of chitinases lead to smaller chitin particles, leading to a change in the specific immune response, eventually resulting in changed cytokine production. This change could lead to a shift in balance between Th1 and Th2 response, reflecting pro- and anti-inflammatory states, with a Th1 response limiting tissue damage in the host and a Th2 response inducing tissue damage (30). This Th1/Th2 balance could make the difference between rat survival and rat mortality in IPA (55-57).

The most important Th1 cytokine is interferon-γ whereas most important Th2 cytokines are IL-4, IL-5, IL-10 and IL-13 (58). Profiles for systemic IL-5 and IL-10 and, partly, pulmonary IFN-γ reported in this thesis on day 1 and 3 but not on day 6 in our IPA model, substantiate this hypothesis, whereas levels of systemic IL-4, IL-13 and IFN-γ and pulmonary IL-4, IL-5, IL-10 and IL-13 did not change significantly during IPA (**Chapter 3, Figure 2**). Thus the data we found are at least party supportive of this hypothesis. However, exact time frames in which cytokine and chemokine levels reach their maximum are unknown and the half-life time of cytokines *in vivo* can be brief. Thus further studies of cytokine profiles generated during IPA are needed to further unravel the role of the balance between Th1/Th2 cytokines in IPA.

CHITINASES IN ANOTHER FUNGAL INFECTION: *MADURELLA MYCETOMATIS* MYCETOMA

In **Chapter 4** we showed that chitinases are upregulated after exposure to *Aspergillus fumigatus*. Since it is one of the major cell wall components of *A. fumigatus*, it is not surprising that exposure to chitin causes a rise in chitin-degrading enzymes. This finding raised the question whether chitinases are upregulated as result of exposure to other sources of chitin as well. Chitin is ubiquitous in nature and is found in many organisms, such as parasites, insects and nematodes (59). In **Chapter 6**, we investigated whether levels of chitinases are also found in individuals suffering from another fungal infection: mycetoma. Mycetoma is a chronic granulomatous infectious disease, resulting in tissue destruction. This invalidating disease is mainly found in tropical areas (60). It can be caused by different micro-organisms, but globally the most common causative agent is the fungus *Madurella mycetomatis*. The fungal agent penetrates the skin and causes granulomatous grains, consisting of fungal material, cement and inflammatory

cells (61, 62). We showed that the grain also contains chitin (**Chapter 6**), which had not been proven previously. In addition to demonstrating the presence of chitin, we also confirmed that both chitotriosidase and AMCase are present in the grain (**Chapter 6**), which had also not been reported previously.

GENETIC POLYMORPHISMS IN CHITINASE GENES

As described above, levels of both mammalian chitinases are increased as a result of invasive fungal infection. The exact role of chitinases in clearance of the fungal material and in infectious diseases in general, is not completely clear. Chitinases are thought to be part of the antiparasitic immune response, since many parasites carry chitin (63, 64). Association between chitinase activity or expression and several diseases was studied previously. (48, 49, 64-98; Table 1).

Chitotriosidase is produced by macrophages and polymorphonuclear neutrophils and can be found in the lungs of mammals as well as in their lacrimal glands, where it has antimicrobial activity (49, 64-67). Increased levels of chitotriosidase are found in Gaucher's disease, a lysosomal storage disease (68) and in asthma (69), but infectious diseases such as candidiasis, malaria and even aspergillosis have also been associated with increased levels of chitotriosidase in serum or plasma (see Table 1)(70-73). Furthermore, expression of chitotriosidase in the stomach was correlated with *Helicobacter pylori* infection (74).

A genetic polymorphism in the chitotriosidase gene, resulting in a decrease in enzymatic activity, was described previously (48, 68, 75, 76). Homozygosity leads to complete inactivity of chitotriosidase whereas heterozygosity for the 24-bp insertion leads to a variable decrease in chitotriosidase activity (67, 77). This polymorphism is found relatively frequently in the healthy population all over the world (68, 78), however with major variation by geographic areas (48, 68, 79-83). Several studies investigated the association between this polymorphism and pulmonary diseases. Heterozygosity for the 24-bp insertion was in some studies, though not in all, associated with asthma (84, 85) and correlation between the polymorphism and clinical outcome in sarcoidosis was not confirmed (86). The association between this polymorphism, resulting in enzymatic inactivity, and pulmonary infectious diseases was not investigated previously.

The other chitinase, AMCase, was also studied previously (47, 51, 63, 65, 74, 87, 99-109; Table 2). AMCase is produced by macrophages and epithelial cells (99). It is found in the stomach (65, 74, 100), tears (101), sinus mucosa (102), saliva (103) and lungs (47, 65, 104) and it is thought to play a role in allergic diseases. Zhu et al showed that production of AMCase is upregulated in Th2 inflammatory responses in the lungs (47). However,

Table 1. Polymorphisms in the gene for chitotriosidase and the associations studied.

Polymorphism in chitotriosidase	Ref	Association described
Rs3831317 (24bp insertion)	48	Associated with increased susceptibility to filarial infection with *Wuchereria Bancrofti* in South Indians
	68	Prevalent in Dutch subjects and in Ashkenazi Jewish subjects, chitotriosidase deficiency is caused by homozygosity of a 24-bp duplication
	77	Prevalent in Europeans, including in Gaucher disease
	79	No chitotriosidase deficiency found in African subjects; high levels of chitotriosidase in acute malaria infection in African children. Suggestion that mutant allele increases the susceptibility to malaria.
	83	Found very frequently in Peruvian Amerindians exposed to chitin-bearing food and enteroparasites
	84	Association between heterozygosity and asthma
	85	Not associated with asthma in children
	86	Not associated with outcome in Sarcoidosis
	87	Associated with decline in FEV1 in African Americans
	88	Not associated with bronchial asthma in Caucasian children
	89	Not associated with atopy in Korean children
	90	Associated with Gram-negative bacteremia in children with AML
	91	Not associated with sepsis or death caused by sepsis in patients with AML
	92	Not associated with chronic disseminated candidiasis in acute leukemic adults
	93	Not associated with filarial infection status, lymphedema of an extremity or hydrocele.
	94	Not associated with coronary artery disease
	95	Not associated with atherosclerosis, however in subjects with athersclerosis higher levels of chitotriosidase were found
	96	Chitotriosidase activity does not correlate with lipid levels; mean levels of enzymatic activity did correlate with allelic dosage
	97	Associated with development of non-alcoholic fatty liver disease
	98	Associated with longevity in Mediterranean
G102S	76	Found in Asians, Africans and Europeans; normal chitotriosidase activity
	77	Prevalent in Europeans, including in Gaucher diseases
Rs1065761	88	Not associated with bronchial asthma in Caucasian children
	89	Associated with atopy in Korean children
Rs2297950	87	Strongly associated with decline in FEV1 in Caucasians
	88	Not associated with bronchial asthma in Caucasian children
Rs2494303	76	Found in Asians, Africans and Europeans; normal chitotriosidase activity
A442G	89	Not associated with atopy in Korean children
G354R	76	Mainly found in Africans; associated with reduced
A442V		chitotriosidase activity
Haplotypes	76	Chitotriosidase deficiency was associated with tuberculosis in European subjects, but not in Asians Chitotriosidase deficiency was associated with less atopic conditions (allergic rhinitis, contact dermatitis, drug or food allergy, asthma) in Asians Chitotriosidase deficiency was associated with less atopic conditions (allergic rhinitis, contact dermatitis, drug or food allergy, asthma) in Asians

also a limitation of chitin-induced eosinophil and basophil influx was reported as a result of AMCase production (51, 105). Mainly associations with allergic diseases such as asthma have been described (47, 63, 106) and AMCase plays a role in the Th1/Th2 responses in the lungs (107) although its exact function remains to be clarified. Several polymorphisms in the gene for AMCase were described and some were associated with asthmatic diseases (see Table 2)(87, 106, 108) Association between infectious pulmonary diseases and altered AMCase activity was not investigated previously.

We hypothesized that the risk for IPA might be influenced by the (in)ability to produce chitinases. As we showed in chapter 3, a combination of chitotriosidase and AMCase is necessary to degrade the fungal cell wall. In **Chapter 5** we investigated 4 polymorphisms resulting in altered chitinase activity in a group of patients at risk for IPA. Probably due to small numbers of patients with IPA we did not find a correlation between decreased chitinase activity and the occurrence of IPA. However, we did find a trend for an association between impaired chitotriosidase activity and an increased risk for IPA. No association was found between incidence of IPA and altered activity of AMCase. We concluded that other factors than chitinase activity seem to play a more important role in the development of IPA. Other polymorphisms that seem to play a role in the development of IPA have been described previously. Polymorphisms in the genes for TLR1, TLR 6 (111), TLR4 (112), mannose-binding lectin (113) and plasminogen (114) were associated with increased incidence of invasive aspergillosis or other invasive fungal infections. Also, polymorphisms in the genes for several cytokines or their receptors were associated with increased incidence of IPA, such as IL-10 (115, 116), IL-1α (117) and CXCL2 (118) and TNF-receptor type 2 (119). For chitinases, the investigated polymorphisms were not associated with increased risk for IPA in our small cohort. Combination of these polymorphisms with other polymorphisms was not investigated, but could reveal a further increase of the risk for IPA. Genetic screening for polymorphisms could identify the population that is at increased risk for invasive fungal infections, but for now the relation between decreased chitinase activity and IPA risk remains to be proven. Van der Velden *et al* reviewed genetic polymorphisms that were associated with invasive mold disease (IMD) and concluded that many polymorphisms are associated with IMD but also concluded that many inconsistencies have been reported (120). They suggest that consistent polymorphisms need to be tested in much larger cohorts, before conclusions can be drawn for their value in risk assessment in infectious diseases (120).

In addition to IPA, we investigated the influence of polymorphisms in the genes for chitinase in patients with *M. mycetomatis* mycetoma (**Chapter 6**). We investigated the same 4 polymorphisms in the genes for chitinases as in **Chapter 5**, in order to assess these polymorphisms as a potential risk factor to develop mycetoma. We found that

mycetoma patients more often have impaired chitotriosidase activity than healthy controls. For AMCase activity, no difference was found between patients and controls.

Apparently, chitotriosidase activity is more crucial in the development of mycetoma than is AMCase. Bierbaum *et al* investigated the presence of polymorphisms in the genes for both chitotriosidase and AMCase in a population at risk for bronchial asthma (88, 106) and found a difference in the association between asthma and both chitinases.

Table 2. Polymorphisms in the gene for AMCase and the association studied.

Polymorphism AMCase	Ref	Association described
Rs3818822	87	Homozygosity associated with lower FEV1 in Caucasians
	106	Associated with increased prevalence of asthma in children and adults
	108	No association with asthma in African American subjects
	110	No direct association with asthma#
K17R	106	Associated with increased prevalence of asthma in children and adults
	110	No direct association with asthma#
Rs12033184	106	No association with asthma
	109	Linkage disequilibrium; no association with asthma for the block of SNPs
	110	No direct association with asthma#
Rs2275253	106	No association with asthma
	108	No association with asthma in African American subjects
	110	No direct association with asthma#
Rs2275254	106	No association with asthma
	108	No association with asthma in African American subjects
	110	No direct association with asthma#
Rs36011905	106	No association with asthma
	110	No direct association with asthma#
Rs2256721	106	No association with asthma
	108	No association with asthma in African American subjects
	110	No direct association with asthma#
G339T	87	Associated with accelerated loss of FEV1 in African Americans
	108	Associated with asthma in African Americans
A531G	108	No association with asthma in African American subjects
Rs4442363	109	All SNPs were in linkage disequilibrium; no association
Rs35042265		the with asthma for block of SNPs based on rs4442363
Rs4546919		
Rs4554721		
Rs11102235		
Rs34698010		
Rs11102234	109	No variation observed in the population investigated
Rs12023459	109	Same prevalence in healthy children and in asthmatic Turkish children

#haplotype-based association analyses with asthma genes (IL-4, IL-13, IL-8, IL-15 and TLR10) showed best evidence for a joint effect on asthma for AMCase and IL-4 and for AMCase and IL-13.

Decreased activity of AMCase was associated with increased prevalence of bronchial asthma, whereas chitotriosidase inactivity was not associated with asthma (88, 106). These studies suggest a difference in function for the two chitinases, while both have the same substrate. Previously it was shown that chitotriosidase is found in a Th1 response, whereas AMCase is part of a Th2 response (121-125). From our study, it seemed that chitotriosidase is essential to skew the immune response towards a Th1-response, resulting in the clearance of the fungal material and preventing a grain to be formed.

Based upon the results of **Chapter 6** and previously published studies, we posit the hypothesis that the balance between a Th1 and Th2 response is crucial in the development of mycetoma (122, 126-130). We showed that impaired activity of chitotriosidase is associated with increased risk for mycetoma. However, other factors also play a role. Several different polymorphisms were shown to influence risk for mycetoma (126, 127, 131, 132). Screening for the chitotriosidase polymorphism could result in identification of a risk group for mycetoma. However, due to costs, screening for a genetic polymorphism is not yet feasible in tropical areas with limited financial supplies such as Sudan, where mycetoma is endemic. Other tests might help out. For example, screening for chitotriosidase activity bypasses the genetic test and could identify individuals with impaired chitotriosidase activity. However, in healthy individuals, levels of chitotriosidase activity are low as well (133). Furthermore, tests for chitotriosidase are not commercially available yet. These problems limit the usability of the genetic polymorphism as screening test in daily use endemic areas such as Sudan. Since the exact role of chitinases is not fully understood, further studies are needed to better understand the function of chitinases in the development of mycetoma.

NOVEL DIAGNOSTIC TECHNIQUES FOR IDENTIFICATION OF *ASPERGILLUS* SPECIES

Diagnosing a fungal infection is usually not easy. Lack of specific symptoms, heterogeneous patient populations and low sensitivity of diagnostic tests challenge clinicians to diagnose IPA in the immunocompromised patient. However, for early start of adequate therapy, it is required to accurately identify the causative agent of the infection, for example for antifungal susceptibility testing.

Over the last years, a novel species of *Aspergillus* was discovered, called *Aspergillus lentulus* (134). With conventional techniques, it is very difficult to distinguish *A. lentulus* from *A. fumigatus*. Studies showed that many isolates of *A. lentulus* have previously been misidentified as *A. fumigatus* (135). The distinction between both fungal species is important, however, since susceptibility for several widely used antifungal agents differs between the two species (136-138). In **Chapter 7** we compare two newer techniques to

identify *A. fumigatus* and *A. lentulus*. We investigated MALDITOF-MS, which was used before in identification of fungi (139-141) and Raman spectroscopy, which already showed its value in identification of bacterial species (142, 143). Both techniques were tested on their ability to correctly identify several isolates of both species. Buskirk *et al* showed that pigmentation of fungi hampers identification by MALDI-TOF MS (144). Conidia of *A. lentulus* and *A. fumigatus* are both darkly pigmented. However, despite pigmentation, MALDI-TOF MS correctly identified 100% of the isolates. Pigmentation did hamper identification of fungi in Raman spectroscopy. Performed on white conidia, Raman spectroscopy correctly identified 78% of fungal isolates. We concluded that MALDITOF-MS is an accurate technique to rapidly identify *A. lentulus*, but Raman spectroscopy is not suitable for daily use due to its lower specificity (**Chapter 7**).

MALDI-TOF MS was confirmed as identification method for fungi and yeasts in several other studies (139, 145-148), but also other novel techniques have been investigated. Most novel techniques are PCR-based. Staab *et al* used PCR-restriction fragment length polymorphism method to distinguish several variants of *A. fumigatus* (149). Furthermore, ITS sequencing of the β-tubulin, calmodulin and rodlet A genes whether or not combined with antifungal susceptibility testing was also used to identify *A. lentulus* (150-152). Finally, Tamiya et al identified *Aspergillus* species based on secondary metabolite profiles (153). The disadvantage of most of these techniques is that they are also culture based and thus require time. In our study, time frame was an important variable in the diagnostic route for both techniques. A reduced diagnostic period enhances early treatment of the patient and improves clinical outcome. Both MALDI-TOF MS and Raman spectroscopy remain culture-based. Culture is time consuming and has a low sensitivity, which are disadvantages. However, based on conventional morphological properties it is still very difficult to distinguish both species. The ideal detection technique would be a rapid method with high specificity and high sensitivity, which can be performed directly on patient materials. Further technical improvement and development of the current techniques could increase the detection rate for fungi in patient material.

NOVEL THERAPEUTIC STRATEGIES FOR INVASIVE ASPERGILLOSIS

Even though several classes of antifungal agents are in use to date, the mortality of IPA remains dramatically high (154, 155). Therefore it is important to not only improve diagnostics (see above), but to also improve the currently available therapeutic strategies. In **Chapter 2, 3 and 4** we discussed the mechanism of action of caspofungin and hypothesized how the clinical outcome of treatment with caspofungin could be improved. Improvement of current antifungal strategies is very important, but the search for novel antifungal agents must also continue. In this context, we investigated a group

of peptides with antifungal properties. Branched histidin and lysin rich peptides (BHKPs) were originally developed as gene delivery agents but were shown in prior studies to have antifungal activity against *Candida* species (156-158). BHKPs are thought to act via a similar mechanism as histatin-5, which also is a histidine-rich peptide, that binds to Ssa1/2 receptor in *Candida* species (159). After binding this receptor, it causes dysfunction of the Trk-1p transporter, resulting in efflux of ATP and other small molecules, resulting in cell death (157).

In **Chapter 8** we tested the *in vitro* antifungal activity of three different BHKPs on several species of fungi and molds, including *Aspergillus* species, *Candida* species, *Cryptococcus* species and *Trichosporon* species. We showed that BHKPs mainly have antifungal activity against *Cryptococcus* species and *Trichosporon* species, but not against *Aspergillus* species and *Candida* species, in contrast to previously published data. Differences in methods (different culture media used) could be responsible for the observed differences in minimal inhibitory concentrations (MICs). These agents could be a new treatment option for infections with *Cryptococcus* species and with *Trichosporon* species but are less suitable for infections with *Aspergillus* species and *Candida* species. More *in vivo* studies are needed to investigate toxic side effects and adverse events of this new category of antifungal agents.

MECHANISM OF ACTION OF CASPOFUNGIN: HYPOTHESIS BASED UPON THIS THESIS

In this thesis we investigated the mechanism of action of caspofungin and its effects on the immune system of the host. It is evident that chitinases play a role in the immune response to chitin-containing fungi. Based on this thesis we developed a hypothesis of how caspofungin and the immune system act synergistically in the clearing of fungal pathogens (see Figure 1). Exposure of *A. fumigatus* to caspofungin causes a shift in cell wall components with increase of chitin and decrease of β-glucan. As a result of the exposure of the host to chitin, the host produces two chitinases, chitotriosidase and AMCase. Caspofungin also unmasks the deeper cell wall layers by releasing galactomannan from its surface. When deeper layers are more exposed, the produced chitinases can better reach chitin polymers in the deeper cell wall layers. Chitotriosidase and AMCase cooperate in the degradation of chitin polymers, resulting in lysis of the fungal cell wall and in smaller chitin particles, which mostly are immunologically inert. The smaller chitin particles shift the immune response towards a Th1 response. The balance between Th1 and Th2 responses is responsible for either clearance of the fungal infection resulting in reduced mortality, or increase of the inflammatory response, ultimately resulting in

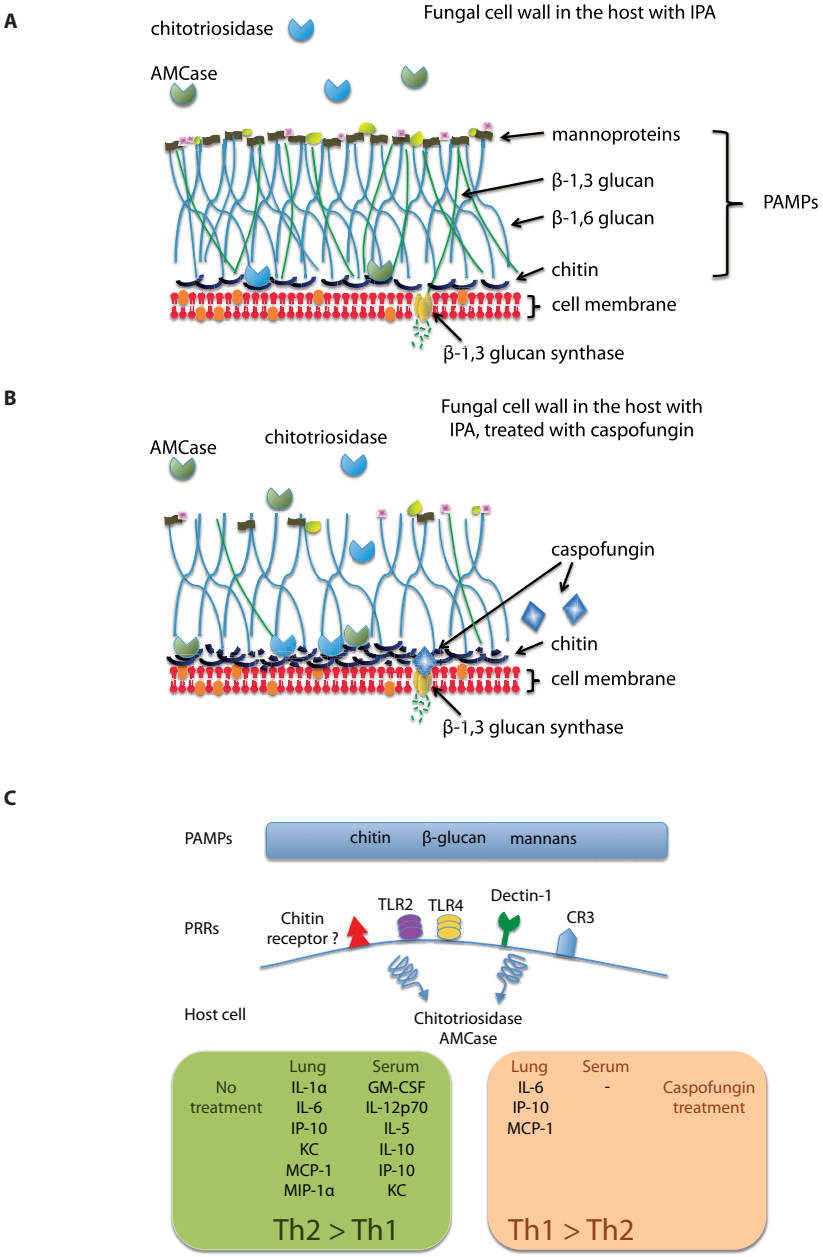

Figure 1.

A. Schematic representation of the fungal cell wall in the untreated host with IPA. Host chitotriosidase and AMCase cannot easily reach chitin, which is found in deeper cell wall layers, and is covered by a superficial layer of mannoproteins.

B. Schematic representation of the fungal cell wall in the caspofungin-treated host with IPA. After treatment with caspofungin, chitin in the fungal cell wall is upregulated and β-glucan synthesis is inhibited. Due to release of mannoproteins, deeper cell wall layers are unmasked. Chitotriosidase and AMCase can better reach chitin and cleave larger polymers into smaller particles.
C. Schematic representation of the changes in cytokine production in different compartments as measured in our animal model of invasive pulmonary aspergillosis. The PAMPs chitin, β-glucan and mannans are recognized by different PRRs, such as TLR2, TLR4 and dectin-1 on the host immune cell. A specific chitin receptor was not described yet, however since chitin was shown to act as a PAMP, the existence of such a receptor is assumed. Stimulation of the PRRs by the specific PAMPs leads to activation of the immune system. Chitotriosidase and AMCase are produced and production of several cytokines is increased in several compartments. Treatment with caspofungin leads to an attenuation of the increase in most cytokines. Levels of these cytokines determine the balance between Th1 and Th2 responses, which ultimately is thought to qualify the outcome in the patient. TLR2 toll-like receptor 2; TLR4 toll-like receptor 4; CR3 complement receptor 3.

death. Treatment with caspofungin shifts the balance towards Th1 response, resulting in clearance of the infection and in survival of the host.

Based upon this hypothesis, it can be questioned whether additional treatment with recombinant chitinases in combination with to caspofungin would result in even better outcomes for the host. After all, addition of certain immune cells, such as monocytes, monocyte-derived macrophages and serum itself were shown to enhance the fungistatic activity of caspofungin (160). Moreover, higher levels of chitinases were found to be protective in murine models of cryptococcosis (161). However, in our murine model of IPA we found a trend for lower chitinases among rats successfully treated with caspofungin, a finding that is possibly due to a much lower fungal load presented to the host system in these caspofungin-treated rats compared to rats left untreated.

More studies are needed to further investigate the combined effects of caspofungin and recombinant chitinases. One suggestion would be to investigate the *in vitro* susceptibility of *A. fumigatus* to combination of caspofungin and recombinant chitotriosidase and recombinant AMCase. When this combination has been proven successful, *in vivo* studies will have to be performed to investigate pharmacokinetics and pharmacodynamics of recombinant chitinases, since these enzymes were not used previously for treatment of mammals. Furthermore, caspofungin treatment could be combined with cytokine therapy in order to influence the balance between Th1 and Th2 cytokines and shift it towards a Th1 response, hopefully resulting in better outcome in the patient.

In summary, chitinases and their role in infectious pulmonary diseases are a promising field of interest and offer possibilities for the improvement in management of fungal infections.

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Nederlandse samenvatting

ASPERGILLUS FUMIGATUS EN INVASIEVE PULMONALE ASPERGILLOSE

Aspergillus fumigatus is een veelvoorkomende sporenvormende schimmel, die zowel buitenshuis als in gebouwen frequent wordt aangetroffen. Sporen worden dagelijks in- en uitgeademd. Mensen met een normaal functionerend immuunsysteem worden niet ziek van deze schimmel, maar bij een niet of minder goed functionerend, of juist overactief immuunsysteem kunnen overgevoeligheid of invasieve schimmelinfecties optreden. Helaas overlijden veel van de patiënten met invasieve pulmonale aspergillose (IPA) nog aan de ziekte.

Het diagnosticeren van een schimmelinfectie is in de kliniek vaak erg lastig. Er is een grote verscheidenheid aan patiënten die risico lopen, en verder zijn de symptomen niet specifiek. Ook de laboratoriumdiagnostiek zou kunnen worden verbeterd. Het kan namelijk lastig zijn om verschil te maken tussen kolonisatie met schimmelsporen (de schimmel rust toevallig even in de longen) en een infectie (de schimmel veroorzaakt lokaal weefselschade). Het vaststellen om welke schimmel het gaat is erg belangrijk, omdat de behandeling afhankelijk is van de schimmelsoort. Daarom is het van belang om zo snel mogelijk het type en subtype van de ziekmakende schimmel te detecteren.

Als de schimmel eenmaal gedetecteerd is, is behandeling ervan de volgende uitdaging. Er is een beperkt arsenaal aan anti-schimmelmedicatie op de markt. Bij IPA is voriconazol de 1^{e} keus. Als de patiënt niet opknapt met voriconazol, kan caspofungin worden gegeven. Caspofungin werkt op de schimmelcelwand en verandert deze van samenstelling. Bij testen met caspofungin in het laboratorium remt dit middel de schimmelgroei pas bij hoge concentraties. Echter, als patiënten worden behandeld met caspofungin in lagere doseringen, knappen ze wel daadwerkelijk op. Het is niet duidelijk waardoor dit verschil tussen *in vitro* en *in vivo* effect wordt verklaard. In dit proefschrift wordt onderzocht hoe verschillende onderdelen van het immuunsysteem van de gastheer betrokken zijn bij het klaren van de schimmelinfectie onder behandeling van caspofungin.

CHITINASES BIJ SCHIMMELINFECTIES

In **hoofdstuk 2** is onderzocht hoe de celwandsamenstelling van *A. fumigatus* verandert door behandeling met caspofungin. Twee belangrijke celwandcomponenten zijn de suikers chitine en β-glucan. Caspofungin remt de aanmaak van β-glucan, waarop de schimmel reageert met extra chitine aanmaak. Deze veranderde celwandsamenstelling geeft een veranderde groei, die ook zonder microscoop goed zichtbaar is. Pas bij heel hoge concentraties caspofungin gaat de schimmel dood. In **hoofdstuk 2** wordt beschreven dat als een β-glucan remmer zoals caspofungin wordt gecombineerd met een chitine remmer zoals nikkomycine Z, synergie optreedt. Beide middelen versterken

elkaar dusdanig dat bij lage concentraties van beide middelen de schimmelgroei wordt geremd. De cellen zien er groter en opgeblazen uit, waardoor ze uiteindelijk kapot gaan. Helaas is nikkomycine Z niet vrij verkrijgbaar dus klinisch onderzoek met deze combinatie middelen is niet mogelijk.

De buitenkant van schimmelcellen bevat structuren (pathogen associated molecular patterns; PAMPs) die kunnen worden herkend door het immuunsysteem van zoogdieren. Chitine en β-glucan zijn voorbeelden van PAMPs. De receptoren om deze PAMPs te herkennen heten pattern recognition receptors (PRRs) en hiervan zijn dectin-1 en TLR-2 en TLR-4 voorbeelden. Elke stimulatie van PRRs door PAMPs leidt bij de gastheer tot een bepaalde reactie met enzymen en signaalstofjes zoals cytokines en chemokines. Als de stimulus verandert doordat de celwandsamenstelling is veranderd, is dus ook te verwachten dat daardoor ook de signaalstofjes veranderen. In **hoofdstuk 3** wordt onderzocht welke cytokines en chemokines veranderen als een rat geïnfecteerd wordt met *A. fumigatus*. In totaal zijn 24 cytokines en chemokines onderzocht, waarbij van een groot aantal gedurende de infectie verhoogd raken. Ook is gekeken naar de verschillen in cytokinerespons tussen onbehandelde ratten, die de infectie niet overleven, en ratten die behandeld zijn met caspofungin, die de infectie allemaal overleven. In grote lijnen bleek de cytokinerespons overeen te komen, met enkele subtiele verschillen. Vanwege kleine aantal dieren die onderzocht zijn, werden geen evidente significante verschillen gevonden.

Hoewel zoogdieren zelf geen chitine kunnen aanmaken, zijn ze wel in staat om chitinases te produceren. Chitinases zijn enzymen die chitine afbreken en die worden aangemaakt na blootstelling aan chitine in bijvoorbeeld schimmels. Er zijn bij mensen 2 chitinases bekend, chitotriosidase en acicid mammalian chitinase (AMCase), met elk hun eigen aangrijpingspunt op het chitine molecuul.

Aangezien caspofungin bij *A. fumigatus* een verhoging geeft van chitine, was het de verwachting dat de chitinase activiteit ook zou toenemen in de gastheer met IPA. In **hoofdstuk 4** is de chitinase respons onderzocht in ratten die blootgesteld waren aan *A. fumigatus*. Een deel van de ratten had een normaalwerkend immuunsysteem en werd niet ziek van de schimmel, maar een ander deel van de ratten was immuun gecompromitteerd en ontwikkelde IPA. Van de laatste groep werd een deel van de dieren behandeld met caspofungin. De activiteit van zowel chitotriosidase als AMCase steeg na blootstelling aan de schimmel in alle ratten. Na enkele dagen daalde de activiteit in de ratten met normaal werkend immuunsysteem. Bij de onbehandelde immuun gecompromitteerde ratten stegen de activiteit van beide chitinases verder tijdens de infectie. Behandeling met caspofungin gaf, in tegenstelling tot de verwachting, juist minder stijging van de activiteit van beide chitinases. Door behandeling met caspofungin is er in

totaal minder hoeveelheid schimmel aanwezig. Uit onze resultaten concludeerden we dat de hoeveelheid schimmel uiteindelijk meer bepalend lijkt voor de chitinase respons dan de celwandsamenstelling.

Bij laboratoriumtesten bleek de combinatie van caspofungin met chitotriosidase of met AMCase de schimmel niet te doden. Wel bleken beide chitinases gelokaliseerd te zijn op de schimmeldraden. Bij aanvullende testen bleek dat de schimmelcelwand kapot ging wanneer de schimmel werd behandeld met caspofungin en de beide chitinases.

Uit bovenstaande paragrafen komt naar voren dat chitinases een belangrijke rol lijken te spelen in de afbraak van chitine van schimmeldraden. Uit eerdere publicaties is gebleken dat bepaalde genetische afwijkingen (polymorfismen) resulteren in een niet-actief chitinase. Dit is bekend voor zowel chitotriosidase als voor AMCase. Voor AMCase zijn ook polymorfismen beschreven die resulteren in een overactiviteit van het enzym. In **hoofdstuk 5** is onderzocht welke invloed deze polymorfismen hebben op de kans om IPA op te lopen. Bij een grote groep mensen met een verzwakt immuunsysteem, en dientengevolge risico op IPA, is gekeken naar de chitinase genen. Bij de mensen die IPA kregen kwamen alle vier de onderzochte polymorfismen even vaak voor als bij de mensen die geen IPA kregen. Hieruit concludeerden we dat verminderde chitinase activiteit het risico op IPA niet verhoogt en dat overactiviteit van AMCase niet tegen IPA beschermt.

Het is niet duidelijk of andere chitine-bevattende schimmels dezelfde reactie geven als *A. fumigatus* in de gastheer wat betreft chitinase productie. In **hoofdstuk 6** is een andere schimmel onderzocht, *Madurella mycetomatis*. *Madurella mycetomatis* is een schimmel die mycetoma veroorzaakt, wat granulomateuze ontstekingen met 'grains' geeft in met name de extremiteiten. De ziekte komt voor in tropische gebieden en geeft pussende ontstekingen die het bot kunnen aantasten. Uiteindelijk leidt dit frequent tot invaliderende amputaties. Een groot deel van de bevolking in bijv. Sudan wordt blootgesteld aan deze schimmel, omdat deze op de grond voorkomt. Echter niet iedereen die eraan wordt blootgesteld, ontwikkelt deze infectie. Daarom is in **hoofdstuk 6** ook gekeken naar de invloed van chitinases. Allereerst is aangetoond dat de *M. mycetomatis* grain chitine bevat. Daarnaast kleurden zowel chitotriosidase als AMCase ook op de schimmel zelf aan, net als bij *A. fumigatus*. Vervolgens is gekeken naar de rol van de hierboven beschreven polymorfismen in de genen voor chitinases. Het vóórkomen van het chitotriosidase polymorfisme kwam significant vaker voor bij patiënten met mycetoma dan bij de gematchte controlepopulatie. Afwezige chitotriosidase activiteit vergroot dus het risico op mycetoma, maar verhoogde of verlaagde AMCase activiteit was niet van invloed. Mogelijk stuurt chitotriosidase de immuunrespons richting het klaren van de

schimmel, wat mede bepalend is voor het niet-ontwikkelen van een chronische infectie met een grain.

NIEUWE DIAGNOSTIEK EN BEHANDELING VOOR IPA

De laatste jaren is een nieuwe species schimmel ontdekt, die qua uiterlijk identiek is aan *A. fumigatus*: *A. lentulus*. Met conventionele diagnostiek zijn deze twee soorten niet van elkaar te onderscheiden. *A. lentulus* is aanzienlijk minder gevoelig voor antifungale middelen dan *A. fumigatus*. Zoals eerder beschreven, is het belangrijk om zo snel mogelijk de veroorzaker van een schimmelinfectie te identificeren. Zo kan de behandeling in een vroeg stadium gericht worden op deze specifieke ziekteverwekker, waarmee de overleving van patiënten verbetert. In **hoofdstuk 7** zijn twee nieuwe technieken vergeleken om *A. fumigatus* en *A. lentulus* te identificeren. Matrix-assisted laser desorption/ionisation time of flight – mass spectrometry (MALDITOF-MS) identificeert micro-organismen op basis van het gewicht van bepaalde celcomponenten. Door met een laser op het micro-organisme te schieten, valt het uiteen in componenten. Deze componenten geven een piekenpatroon van massa's, waarmee het micro-organisme geïdentificeerd kan worden. Deze techniek is eerder bruikbaar gebleken voor identificatie van schimmels. Raman spectroscopie is een optische techniek, die micro-organismen identificeert op basis van lichtverstrooiing. Elke celcomponent in een micro-organismen geeft een bepaalde verstrooiing van licht. Het spectrum dat hiermee wordt gegenereerd is specifiek genoeg gebleken om bacteriën te identificeren. Schimmels zijn echter nooit getest.

In **hoofdstuk 7** zijn 16 *A. lentulus* isolaten en 16 *A. fumigatus* isolaten geblindeerd getest met beide technieken. Voor Raman werden alleen betrouwbare piekenpatronen gemeten als de schimmels op een ander kweekmedium groeide, waarmee sporen ontkleurd waren. Raman spectroscopie identificeerde 78% van de isolaten correct in ca. 100 uur, terwijl MALDITOF-MS 100% correct kon identificeren in ca. 50 uur. Hieruit concluderen we dat MALDITOF-MS een geschikte techniek is om in de dagelijkse praktijk *A. lentulus* van *A. fumigatus* te onderscheiden. Raman spectroscopie is minder geschikt vanwege de lagere nauwkeurigheid en de langere tijdsduur tot identificatie.

Tot slot is een nieuwe groep medicijnen getest, die mogelijk tegen IPA zou kunnen werken. Branched histidine- en lysine rich peptides (BHKPs) zijn peptides die oorspronkelijk zijn ontwikkeld voor gentherapie. Ze blijken ook antimicrobiële eigenschappen te hebben, waarbij groei bijv. van de gist *Candida* geremd wordt. In **hoofdstuk 8** is de antifungale werking van drie van deze peptides getest. Verschillende groepen schimmels en gisten zijn in het laboratorium getest op gevoeligheid voor verschillende concentraties van de drie peptides. Het bleek dat de vier geteste *Aspergillus* species en de twee *Can-*

dida species niet gevoelig zijn voor de BHKPs. In tegenstelling tot *Aspergillus* en *Candida* bleek de groei van de gisten *Cryptococcus* en *Trichosporon* wel geremd te worden door de BHKPs. Van de drie geteste peptides bleek H2K4b de sterkste anti-microbiële werking te hebben. Voor de behandeling van IPA zijn deze middelen niet geschikt, maar bij gistinfecties zouden ze mogelijk wel effectief kunnen zijn. Gegevens over toxiciteit en over afbraak in het menselijk lichaam zijn niet bekend dus dit zou onderzocht moeten worden voordat deze groep medicijnen op de markt kan komen.

Samenvattend beschrijft dit proefschrift een aantal factoren van het immuunsysteem die bijdragen aan het klaren van de schimmelinfectie IPA. Met deze informatie hopen we IPA beter te begrijpen en met name de reactie van de gastheer die behandeld wordt met caspofungin. Door beter begrip van hoe caspofungin werkt, hopen we de effectiviteit van de behandeling te vergroten, om zo tot een betere klinische uitkomst bij patiënten te leiden.

Dankwoord

Curriculum Vitae

List of publications

PhD portfolio

DANKWOORD

En dan is het zover, je proefschrift is af. Het was een flinke klus, met hier en daar meer vertraging dan gewenst, maar toch is het uiteindelijk af gekomen. Dit geheel was niet mogelijk geweest zonder ontzettend veel steun van de mensen om mij heen.

Prof. dr. Verbrugh, beste Henri. Het zit erop! Geen meetings meer waarin ik uit de doeken doe hoe ver ik ben gekomen, tussen de opleidingsmomenten door. Fijn dat je als promotor, na het vertrek van Alex, meer verdieping richting de schimmels hebt willen zoeken. Ik zal ons fietstochtje op de tandem bij de Veluwe niet gauw vergeten… Bedankt voor het kritisch doorlezen van mijn stukken en voor de opbouwende kritiek. Het product is prachtig geworden.

Dr. van de Sande, beste Wendy. Wat heb ik geboft met jou als copromotor! Super dat je mijn stukken altijd zo snel had nagekeken. Dank voor de vrijheid die je me gaf tijdens mijn promotie, maar ook voor de ruimte die ik kreeg tijdens het starten van mijn opleiding. Dit boekje is ook jouw boekje!

Prof. dr. dr. van Belkum, beste Alex. Zonder jou was ik nooit begonnen aan dit project. Bedankt voor het vertrouwen dat je in mij had, om een arts een lab-promotie te laten starten. Ondanks dat ik er wat langer over heb gedaan dan gemiddeld heb ik je hopelijk niet teleurgesteld. Veel dank ook voor de gastvrijheid in la Balme-les Grottes en voor de mooie publicatie die daaruit is voortgekomen.

Prof. dr. Mouton en prof. dr Netea, veel dank voor het plaatsnemen in de kleine commissie en voor het beoordelen van mijn proefschrift. Prof. dr. Hoogsteden, veel dank voor het uitvoeren van de secretariële taken in de kleine commissie.

Dr. Bakker-Woudenberg, beste Irma. Wat ben ik blij dat ik jouw expertise van dichtbij heb mogen meemaken. De besprekingen werden van serieus in de beginjaren uiteindelijk een steeds gezelliger weerzien nadat mijn labtijd erop zat. Je was altijd kritisch en toch opbouwend. Jouw tekstuele handigheid zal me nog lang bijblijven. Bedankt voor je toewijding maar vergeet af en toe niet aan jezelf te denken!

Jasper! Jasper… Wat moet ik hier nu schrijven. Woorden schieten tekort… Het was voor mij direct duidelijk dat jij mijn paranimf zou zijn. Bedankt voor je mentale ondersteuning en morele support tijdens mijn promotie, zowel op als buiten het werk. En tsja, die eeuwige gewilligheid om de slachtofferrol aan te nemen waardeer ik ook! Respect ook voor

jouw volharding; net als met de marathon: het duurt wat langer, maar je komt er écht. Succes met jouw laatste stukkie!

Char, chica! Top dat jij het vandaag van zo dichtbij kon meemaken. Na onze gezellige start op unit 28 was jij de aangewezen 2e paranimf. Fijn dat je altijd zo enthousiast bent. Nijmegen is wel ver, maar het wereldje is klein. Bedankt voor de vele gezellige borrelmomentjes, je steun tijdens de opleiding en de gezellige dates op en na het werk. Veel succes met je eigen onderzoek, dank dat ik jouw promotor even mocht lenen vandaag.

Prof. Falcone, dear Franco. Thanks for your contribution to Chapter 4 of this thesis. Even though we never met, the enthousiasm in your emails worked contagious and inspired me to continue writing and submitting. Thank you for the cooperation.

Alle overige co-auteurs van de publicaties in dit proefschrift: veel dank voor jullie bijdrage en kritische blik op de manuscripten.

To all other co-authours: thank you for your contribution to the publications in this thesis.

Beste collega's van MMIZ, ik kwam, ik ging en uiteindelijk promoveerde ik. Hadden jullie het nog verwacht? ☺ Het waren gezellige jaren en ook na 3,5 jaar was het een warm weerzien. Bedankt hiervoor! Speciale dank gaat uit naar de onderstaande mensen:

Marian, wat een werk heb je verzet voor mijn dierexperimenten. Dank voor je begeleiding en voor je begrip voor mijn klinische, niet-altijd-lab-blik.

Gerjo, had ik met iemand beter een labtafel kunnen delen dan met jou? Jouw stickers boven mijn bench maakten het leven draaglijker. Dank en succes met de laatste efforts voor jouw boekje.

Jeannine, mede-schimmel-promovendus. Dank voor de gedeelde smart! Veel succes met de laatste loodjes van je eigen promotie. Je komt er heus!

Kimberly, veel dank voor het draaien van ongeveer 382.479 PCRs.

Alle andere postdocs die mij voorgingen, maar ooit tegelijk met mij promovendus waren.. (Rogier, Astrid, Suzanne, Sanne, Wendy Ka, Saskia en vele anderen) Het is toch nog af gekomen! Dank voor jullie gedeelde-smart-is-halve-smart-mentaliteit door de jaren heen, de gezelligheid tijdens het werk en natuurlijk de R&D borrels.

Michiel, dank voor je technische blik op mijn (ICT-)problemen gedurende mijn promotietraject. Fijn dat je als altijd voor me klaar stond. Succes met jouw laatste stuk.

René, alweer een tijdje weg, maar de koffiemomenten zitten nog vers in mijn geheugen! Te gek dat je met mij als eerste wilde investeren in een nieuwe koffiemachine.

Willem vL, top om met jou samengewerkt te hebben. Het hoekje met de scheur achterin de L-vleugel was toch wel een erg gezellig hoekje. Dank voor het mogelijk maken van hoofdstuk 7 en voor je ondersteuning met Raman.

Loes en Hans, dank voor jullie hulp bij het maken en bewerken van de vele foto's voor mijn artikelen. Jullie hulp was onmisbaar.

Carla, jouw keuken-inspanningen waren voor mij als schimmel-promovendus de hemel op aarde. Ik hoefde het maar te zeggen of er stond alweer een nieuwe batch met SAB-platen klaar. Superveel dank voor jouw ondersteuning in de afgelopen jaren.

Alle analisten en andere medewerkers van MMIZ: bedankt voor de gezellige periodes die ik op de afdeling heb gehad.

RopaRun team 27, wat tof dat ik mee mocht doen, al was het geblesseerd het 1e jaar. Fijn dat ik een herkansing kreeg. Nog nooit heeft de tocht van Hamburg naar Rotterdam zo lang geduurd, maar het was het zeker waard. Gouden herinnering! Gerard en Ad, aan jullie een extra bedankje voor het begeleiden van de zwaarste 10 kms uit mijn leven! Toppers!

Minke en Christa, thanks voor de afleiding die jullie met enige regelmaat boden. We houden de etentjes, koffiemomenten en de wijntjes er toch wel in? Mink, succes met jouw laatste loodjes. Ook jij zal er zeker komen!

Meiden van 24, dank voor het delen van alle promotieleed in de afgelopen jaren! Zonder jullie was het heel anders geweest. Fijn dat ik altijd bij jullie mocht klagen. Dank voor de gezellige momentjes om de promotie-stress even te kunnen vergeten. Het klagen wordt zeker minder!

Amphia meiden, Eefje, Sophie, Eri-Jo en Charlotte, wat een top-collega's in Breda! Jullie zorgden voor voldoende ontspanning tussen werk en proefschrift door. Gelukkig zien we elkaar ook buiten Breda regelmatig nu en hebben we nog flink wat mooie feestjes voor de boeg met elkaar. Ik heb er zin in! PS bij wie staat de beker nu?

Juul, Jacq en Chris (+4 natuurlijk). Vanaf nu wordt alles beter… Het was altijd maar weer die promotie en chagrijnig gedoe van mijn kant. Nu is het klaar. Dit jaar is een feestjaar en dit is deel 1. In Lissabon wordt het super! Fijn dat jullie er zijn en dat ik er voor jullie kan zijn wanneer dat nodig is.

Dirk en Margot, de laatste vier jaar ben ik natuurlijk veel met jullie zoon bezig geweest maar ook met dit lange project. Het is uiteindelijk iets moois geworden; nu het af is, wordt het tijd voor een nieuwe hobby denk ik. Superveel dank voor jullie begrip en in-

teresse voor mijn bezigheden van afgelopen periode. En natuurlijk voor de open armen waarmee ik sinds jaar en dag word ontvangen!

Oudersch, daar is het dan... Een beetje voor papa maar ook voor mijzelf. Het was een drukke tijd, waarin ik regelmatig gefrustreerd moest uitleggen dat 'het bij geneeskunde promoties echt heel anders gaat tegenwoordig dan bij rechten in de jaren '70'. Sorry voor alle kortaf-antwoorden, frustraties en het regelmatige zuchten, en dank voor het begrip en de ondersteuning, in alle vormen. Mama, het enthousiasme mag af en toe iets worden beteugeld maar wordt desondanks erg gewaardeerd. Deel 1 vandaag, deel 2 in september!

Bob, lieve Bob. Wat is mijn leven veranderd sinds ik in naar het Amphia kwam. Het gelukkige toeval kon niet beter uitpakken voor ons allebei. Fijn dat je steeds kritisch wilde meekijken, meelezen en aanpassen in mijn teksten. Jij hebt wel het meeste begrip getoond van allemaal. Gelukkig kan ik soms iets terugdoen in jouw presentaties, al is het wat gezever (soms dan!). Nu een klein feestje, in september een groots feest. Daarna wil ik die wijncursus wel overwegen… You're the best!

CURRICULUM VITAE

Patricia Eline Bernadette Verwer was born on 21st of July, 1983 in 's Gravenhage. After secondary school (College Hageveld te Heemstede), in 2001-2002 she studied Policy & Health Management (Beleid & Management van de gezondheidszorg, first-year examination passed) at Erasmus University, Rotterdam, after not being selected for medical school due to the lottery system. In 2002, she was nevertheless selected for medical school by 'decentrale selectie' at Erasmus MC, Rotterdam. During studies she participated in several education-oriented committees. She completed the internships at St. Elisabeth Ziekenhuis te Tilburg. In 2007 Patricia went to Perth, Australia, for a Master's thesis concerning *Staphylococcus aureus* colonization in healthcare workers, under supervision of Tom Riley and Geoff Coombs. This work was presented on International Symposium on Staphylococci and Staphylococcal Infections (ISSSI 2008) in Cairns, Australia. In 2008 she graduated from medical school and started working as resident at St. Elisabeth Ziekenhuis. Patricia was invited by prof. van Belkum for a PhD study at the department of Medical Microbiology & Infectious Diseases at Erasmus MC. In 2009 she started her PhD on *Aspergillus fumigatus* infections, under direct supervision of Wendy van de Sande. During her PhD Patricia participated in several courses organized by the Postgraduate School of Molecular Medicine. Furthermore the research results were presented on several scientific meetings, including those of International Conference on Microbial Resistance and of Dutch Association for Medical Mycology (NVMMy). She also participated in the PhD day committee in 2010 and 2011. Prof. Verbrugh took over supervision of the PhD after prof. van Belkum switched jobs.

In 2012 Patricia started the clinical specialization of Internal Medicine. From Erasmus MC she found a position in Amphia Ziekenhuis, Breda, where she worked from 2012 to 2015. In 2015 she came back to Erasmus MC for the final 3 years of the specialization. During the specialization Patricia had extra interest for Infectious Diseases and she was trained for 8 months in this discipline. After her PhD Patricia will further differentiate into Acute Internal Medicine, with particular attention for Infectious Diseases.

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- Verwer PE, Robinson JO, Coombs GW et al. Prevalence of nasal methicillin-resistant *Staphylococcus aureus* colonization in healthcare workers in a Western Australian acute care hospital. Eur J Clin Microbiol Infect Dis 2012. 31:1067-1072.

- Verwer PE, Woodle MC, Boekhout T et al. *Cryptococcus* and *Trichosporon* spp. are susceptible *in vitro* to branched histidine- and lysine-rich peptides (BHKPs). J Antimicrob Chemother 2011. 66:1649-52.

PHD PORTFOLIO

Summary of PhD training and teaching activities

Name PhD student: Patricia Verwer
Erasmus MC Department: MMIZ
Research School: MolMed

PhD period: april 2009-june 2016
Promotor: H.A. Verbrugh
Supervisor: W.W.J. van de Sande

1. PhD training	Year	Workload (hours/ ECTS)
General academic skills		
Biomedical English Writing and Communication	2010	2 ECTS
Organisation PhD day 2010, 2011	2010, 2011	1 ECTS
Research skills		
Course Biostatistics for clinicians	2010	1 ECTS
Course Photoshop CS3 workshop	2010	0,25 ECTS
Course Basic introduction course on SPSS	2010	0,5 ECTS
Course Research Management for PhD students and Postdocs	2010	1 ECTS
In-depth courses (e.g. Research school, Medical Training)		
Course Biomedical Research Techniques	2009	0,3 ECTS
COIG cursussen: Klinische farmacologie, Water & zout, Moleculaire biologie, Klinische genetica	2013, 2014 (2×), 2015	4 ECTS
5th Rotterdam course in electrolyte disorders	2013	8h
FCCS cursus (Ede)	2014	0,5 ECTS
(Inter)national conferences		
NVMM Wetenschappelijke voorjaarsvergadering: poster presentation (Papendal)	2010	1 ECTS
International Conference on Antimicrobial Resistance: oral presentation (Valladolid, Spain)	2010	1 ECTS
NVvMy wetenschappelijke Najaarsvergadering: oral presentation (Utrecht)	2010	0,5 ECTS
NVVM Wetenschappelijke voorjaarsvergadering: poster presentation (Papendal)	2011	1 ECTS
NVvMy Wetenschappelijke najaarsvergadering 2011: oral presentation (Utrecht)	2011	0,5 ECTS
Wetenschapsmiddag Amphia ziekenhuis Breda	2012, 2013	8h
Internistendagen	2013, 2014, 2015, 2016	3 ECTS
Afscheidssymposium prof. Verbrugh: Golden Oldies - staphylococcen	2015	5h

6th European Congress of Clinical Microbiology and Infectious DIseases (Amsterdam)	2016	1 ECTS
Seminars and workshops		
Workshop Finding literature I & II, workshop Endnote (Medical Library)	2009	4,5h
Workshop Negotiation (VVAA)	2010	2,5h
3rd Annual symposium on Host Defence Peptides: oral presentation	2010	0,5 ECTS
Workshop Allemaal beestjes; infectieziekten in de huisartsenpraktijk	2011	5h
MolMed & NVMM: Microbial pathogenesis	2011	0,25 ECTS
Bijscholingen afdeling MMIZ HIV, parasieten, antibiotica, SOA, darminfecties	2009, 2010, 2011	9h
Klinische avond Interne Geneeskunde (Nal)	2012, 2013 (2×), 2015	12h
DOO Gezondheidsrecht Amphia ziekenhuis	2013	4h
DOO Samenwerking Desiderius school	2013	8h
DOO Kwetsbare ouderen Amphia ziekenhuis	2013	8h
DOO Financiering van de zorg en de arts van straks	2014	8h
Didactic skills		
DOO Teach the Teacher II	2015	1 ECTS
Other		
PhD Day Erasmus MC	2009, 2010, 2011	15 h
Journal Club / Research besprekingen MMIZ	2009, 2010, 2011	2 ECTS

2. Teaching activities	Year	Workload (hours/ ECTS)
Lecturing		
Master Infection & Immunity: Summercourrse II	2010, 2011	8h
Supervising practicals and classes		
Curriculum Erasmus Arts; 2e-jaars onderwijs geneeskunde	2010, 2011	3 ECTS
Klinisch redeneren Bachelor I (3 sessies)	2015, 2016	9h
Supervising HLO trainee		
C.C. Notenboom; sept 2011 – jan 2012	2011	5 ECTS
Total		114h
		30,3 ECTS