General introduction and outline of the thesis

The unprepared mind cannot see the outstretched hand of opportunity.
(Alexander Fleming)

INTRODUCTION

An invasive fungal disease (IFD) is a life-threatening infection that is almost exclusively diagnosed in the immunocompromised host. IFD can be divided into moulds (hyphae forming fungi) and yeasts (strings of connected budding cells forming pseudohyphae). While the most common yeast infection in human is caused by *candida* species, the most common invasive mould infection is caused by *Aspergillus species* and called invasive aspergillosis (IA). Patients with haematological malignancies who are treated with intensive chemotherapy and haematopoietic stem cell transplant recipients are most prone to develop IA. Incidence rates of IA vary substantially and depend on host and environmental factors but also the modalities of stem cell transplantation as well as the use of antifungal prophylaxis. The patients at highest risk are patients with a newly diagnosed acute myeloid leukaemia (AML) undergoing remission induction chemotherapy and allogeneic stem cell transplant recipients who need systemic immunosuppressive therapy for graft-versus-host disease. Without prophylaxis the incidence of IA in these populations can be as high as 10-20% (1-3). IA does not only lead to a higher overall mortality and morbidity but also to higher medical costs (4). The case fatality rate of IA is estimated to lie between 20-38% 6 to 12 weeks after diagnosis (5). Therefore, optimizing the management of IA is key in order to reduce the burden of this devastating complication in the immunocompromised host.

For more than 15 years voriconazole, a drug of the triazole class, has been the recommended treatment for this life-threatening infection after a pivotal randomized trial showed an improved survival with voriconazole compared with amphotericin B deoxycholate. However, also with voriconazole the overall 6-week mortality is still unacceptably high at 25-30% (6). Another strategy in the management of IA is prevention with antifungal prophylaxis. The European Conference on Infections in Leukaemia-5 guideline recommends antimould prophylaxis when the incidence of mould infections is high (7). Firm criteria for what constitutes “high risk” are lacking but it has been proposed that subpopulations with >8-10% fall into this category. Unfortunately reliable data on the exact local prevalence of mould infections are often lacking (3).

A troublesome emerging problem in patients with IA is the increasing incidence of triazole-resistant *A. fumigatus*. Although limited by numbers, case series have demonstrated that the overall mortality of patients infected with triazole-resistant *A. fumigatus* becomes very high (50-88%) (8, 9). Remarkably, from a global perspective the highest prevalence of triazole resistance has been documented in the Netherlands. It increased from 0% before the year 2000 to 5.3% in 2009, and further increased to 15% in 2018 (8, 10). More recently, triazole resistance was observed in 5% of IA cases in Belgium as well and in 2017 researchers from the Erasme hospital in Brussels even reported a prevalence of 13% (11, 12). Different azole-resistant IA cases have been

described globally but resistance rates vary substantially between geographic regions and between hospitals (13).

This thesis focuses on risk factors for and the diagnosis of invasive aspergillosis. Additionally, the management of azole-resistant aspergillosis is addressed.

AZOLE-RESISTANT ASPERGILLOSIS: OUTCOME AND TREATMENT

IA is mostly, although not exclusively, caused by *Aspergillus fumigatus*. As previously mentioned, azole-resistant *A. fumigatus* strains are an emerging global problem and complicate the management of this infection enormously (13). Azole-resistance is mostly caused by a mutation in the *Cyp51A* gene that encodes for the lanosterol 14α-demethylase, the target enzyme for azoles. Two mutation combinations in this *Cyp51A* gene, TR_{34}/L98H and TR_{46}/T289A/Y121F, account for more than 80% of the mutations conferring resistance in the Netherlands (14, 15). These mutations are assumed to have an environmental origin caused by agricultural use of azole fungicides (16-18). Case series indicate that IA caused by azole-resistant *Aspergillus* is associated with very high mortality rates of 50-88% (8, 9). Until now, case series have included very few patients and preclude a reliable estimation of the impact of azole-resistance on mortality. Furthermore, studies in which the outcome of patients infected with a triazole-susceptible or a triazole-resistant *A. fumigatus* is compared are lacking. Therefore, a 5-year retrospective cohort study (2011-2015) was performed to compare the mortality between patients diagnosed with a voriconazole-susceptible and a voriconazole-resistant IA. **Chapter 3** describes the results of this study.

Detection of azole-resistant aspergillosis is challenging. First, a positive fungal culture is required to allow for the use of conventional phenotypic resistance testing. However, in the vast majority of IA cases no positive culture can be retrieved. Second, phenotypic susceptible testing according to internationally agreed methods is almost exclusively done in mycology reference labs and is thus time-consuming. Recently, the clinical validity and relevance of PCR-based susceptibility testing was demonstrated using a commercially available multiplex qPCR: i.e. the AsperGenius© qPCR. Besides detecting the presence of *Aspergillus* DNA, this qPCR allows to detect the two most frequent resistance-associated mutations (TR_{34}/L98H and TR_{46}/T289A/Y121F). Chong and colleagues evaluated the diagnostic performance of this qPCR in 201 patients showing a sensitivity and specificity of 89% and 89% compared with galactomannan and culture results as the gold standard. In addition, this study showed that response to voriconazole therapy was poor, when it was given to patients infected with an azole-resistant *A. fumigatus* strain (9). There are still several open questions to be answered following these studies. First, how the daily use of this qPCR impacts the management and thus

outcome of patients that are suspected of having an IA remains to be demonstrated. In particular, it remains to be seen what the outcome is of patients in which this qPCR is used to guide antifungal therapy. Does the immediate switch from a triazole to another antifungal drug as soon as resistance is documented by PCR reduces the overall mortality compared to the high mortality described above?

To get a reliable picture of the fungal infection management landscape in the Netherlands and in particular in the context of increasing triazole-resistance, a meeting was organized with haematologists, infectious disease physicians and microbiologists from all academic university hospitals in The Netherlands. A survey questioned the prophylactic, diagnostic and therapeutic strategies regarding IFD in all academic centres. The results were processed and during a consensus meeting the protocol for a prospective multicentre study was developed and implemented as the AZOle Resistance MANagement study (AZORMAN) (NCT03121235). The process and rationale of this study are described in **chapter 2**. In this study, a standard diagnostic and therapeutic protocol for IA was agreed upon to be used for patients with an underlying haematological disease who present with a new pulmonary infiltrate and for whom the treating physician decides to order a diagnostic bronchoscopy. The primary objectives of the study are: (1) To improve the outcome of patients infected with azole-resistant *A. fumigatus* by facilitating the early detection of RAMs and with this, earlier initiation of the most appropriate therapy and (2) To monitor the prevalence of IA due to *A. fumigatus* strains carrying the TR_{34}/L98H and TR_{46}/T289A/Y121F RAMs in the Netherlands, in particular in culture-negative patients. Indeed, previous studies have based prevalence estimates on culture positive cases of IA only and this may lead to a biased estimate of the prevalence.

This multicentre prospective study currently running in 11 haematology centres in the Netherlands and Belgium started in 2017 and as of October 2019 recruited more than 2/3 of the projected 280 patients. In **chapter 9** preliminary results from the AZORMAN study are presented.

A report of an international consensus meeting on the management of infections caused by azole-resistant *Aspergillus fumigatus* was published in 2015. The experts recommended a switch from voriconazole to liposomal-amphotericin B in confirmed azole-resistant aspergillosis (19). Guidelines advocate that the duration of antifungal treatment should depend on clinical response, degree of immunosuppression and response on imaging (20). However, liposomal-amphotericin B can only be administered intravenously and has obvious toxicity limitations (kidney failure, electrolyte disturbances). Therefore, the treatment of azole-resistant IA is logistically challenging and costly as most of the patients will stay hospitalized for the daily intravenous administration of liposomal-amphotericin B as there are no validated oral step-down treatment options for patients with azole-resistant IA. In the AZORMAN-study (see **chapter 2** and

9) two options are suggested as possible step-down therapy for azole-resistant aspergillosis. These are liposomal-amphotericin B given intravenously thrice weekly rather than daily at a dose of 5mg/kg or a treatment with posaconazole tablets while targeting high serum trough levels 3-5mg/L. The latter strategy can only be considered when the minimum inhibitory concentration (MIC) of posaconazole of the azole-resistant *A. fumigatus* strain is below 2mg/L. Furthermore, these options should only be considered for patients showing clinical and radiological improvement with daily treatment with liposomal-amphotericin B. In **chapter 4**, we describe the rationale for the use of high-dose posaconazole (HD POS) targeting high serum trough levels and describe our experience with this strategy regarding safety and efficacy. The long terminal half-life of LAmB suggests that intermittent dosing could be effective, making the application of outpatient antifungal therapy (OPAT) possible. In **chapter 5,** together with colleagues from Leiden and Leuven, we describe our experience with intermittently dosed liposomal-amphotericin B in the outpatient setting for the treatment of invasive fungal infections.

The most devastating form of IA is haematogenic dissemination of this fungus to the brain. Brain infections with *Aspergillus* have a very high mortality and survivors are left with at least some neurological deficit (21). Although the chances of survival have improved since voriconazole became available, azole-resistant *A. fumigatus* strains now turn back the clock to the amphotericin B era. Few cases of central nervous system (CNS) aspergillosis caused by azole-resistant *Aspergillus fumigatus* have been reported, but almost always with a fatal outcome (19). Most patients were treated with combination antifungal therapy. Given the dismal prognosis of cerebral infections with azole-resistant *A. fumigatus* and the lack of antifungals with activity against azole-resistant *A. fumigatus* that adequately penetrate the brain we describe our experience with the use of intraventricular liposomal-amphotericin B (L-AmB) on top of systemic antifungal therapy in 3 patients in **chapter 6.**

DIAGNOSIS OF INVASIVE ASPERGILLOSIS

The strength of a diagnosis of IA is currently reported according to the revised definitions of the European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) (22). IA is categorized into proven, probable and possible IFD. A proven diagnosis requires histopathologic evidence of fungal invasion. A diagnosis of probable IA is based on the presence of a combination of host factors, clinical features and a positive mycology test. A diagnosis of possible IA is made in the presence of host factors and clinical features but in the absence of mycological criteria (23). To fulfil mycological criteria a direct test or indirect test has to be present. Direct tests are

the detection of fungal elements or culture positive for *Aspergillus species*. Indirect tests are the presence of antigen or cell-wall constituents like galactomannan antigen (GM) or beta-D-glucan (24). Despite the fact that PCR for the detection of *Aspergillus* in human specimens exists for almost three decades, the technique was not included in the EORTC/MSG consensus definitions for diagnosing IFD because of the lack of standardisation (25). A good step towards standardization is the use of a commercially available PCR like the aforementioned AsperGenius© qPCR. Although this test was retrospectively validated (9, 26), large prospective studies investigating its real-life added value and validity by using the PCR in different laboratories are lacking. The interim results of a first prospective and ongoing study are described in **Chapter 9**. Above, we described the troublesome emergence of azole-resistant IA. Yet, mixed infections with azole-susceptible and azole-resistant strains of *A. fumigatus* have been described in the past by demonstrating the presence of two different *A. fumigatus* strains with two different susceptibility profiles with the use of conventional culture based methods (27). However, many if not the majority of cases of IA that physicians are confronted with are culture-negative. In **chapter 8,** we describe three patients infected with an azole-susceptible and azole-resistant *A. fumigatus* and in whom, for the first time, the mixed infection was demonstrated by *cyp51A* PCR amplicon melting curve analysis using the AsperGenius© assay.

Galactomannan antigen detection and detection of Aspergillus DNA are labour intensive diagnostic tests with turnaround time of at least 24h to 72h as they are mostly performed in batches with 96 well plates. A bed-side point of care test is lacking but also a rapid and easy to perform test that can be used in small microbiology labs is lacking as well. A newly CE-marked later flow device (LFD) may be such a test. It consists of a self-contained immunochromatographic assay using a mouse monoclonal antibody (JF5) for the detection of an extracellular glycoprotein released by *Aspergillus* during active growth (28). In the study described in **chapter 7** and performed in collaboration with the University Hospitals Leuven and coordinated by dr. T. Mercier, we evaluate this test on bronchoalveolar lavage fluid (BALf) collected from adult haematology patients from 4 centres in The Netherlands and Belgium.

INFLUENZA-ASSOCIATED ASPERGILLOSIS

For almost a century, influenza has been known to set up for bacterial superinfections, but recently patients with severe influenza admitted to ICU were also reported to develop invasive pulmonary aspergillosis (29, 30). As these reports were almost exclusively single centre-based and limited to a single influenza season, several important questions regarding the epidemiology of influenza-associated invasive aspergillosis

(IAA) remain unanswered. Therefore, we aimed to measure the incidence of invasive pulmonary aspergillosis over several seasons in patients with influenza pneumonia in the intensive care unit (ICU) and to assess whether influenza was an independent risk factor for invasive pulmonary aspergillosis. The results are presented in **chapter 10.1**. Furthermore, we evaluated if the higher mortality of patients with influenza-associated aspergillosis in the ICU can be attributed to the *Aspergillus* superinfection in se or if it is just a marker of overall disease severity. Therefore, we also performed a mortality analysis on our influenza cohort of 432 patients admitted to the ICU with influenza (see **chapter 10.2**).

AZOLE-ECHINOCANDIN COMBINATION THERAPY FOR INVASIVE ASPERGILLOSIS

As previously mentioned, triazoles like voriconazole or isavuconazole are the recommended treatment options for IA (6, 20, 31). Still, mortality remains unacceptably high at 25-30%. Azoles block the synthesis of ergosterol, a part of the fungal membrane while antifungals from the echinocandin class block the synthesis of Beta-D glucan, a component of the cell. Both drugs may work synergistically as suggested in vitro studies and neutropenic animal models (32, 33). These observations led to the performance of a clinical trial comparing the efficacy of voriconazole with or without anidulafungin, an echinocandin, in a population with haematological malignancy (34). In this trial 6-week mortality was 30% lower in the group treated with combination antifungal therapy (19.3%) versus monotherapy (27.5%) but was not statistically significant (p=0.09%). This is the reason why combination therapy has not been adopted by current guidelines. A second clinical trial is needed to confirm these promising finding. In 2019, following a study proposal by dr. B. Rijnders and Prof. dr. J. Maertens submitted to BeNeFit a grant was awarded to implement such a clinical trial in 25 haematology centres in the Netherlands and Belgium. BeNeFit is a new collaboration between Belgium (KCE) and the Netherlands (ZonMW) in order to support large pragmatic intervention trials. The writing of the study protocol was initiated and coordinated by dr. B. Rijnders and drs. A. Schauwvlieghe and can be found in **chapter 11**.

SUMMARY

Several studies were performed to investigate the incidence, mortality, risk factors and diagnostics of IA. **Chapter 3** focusses on mortality of azole-resistant IA. **Chapter 2** describes the design and rationale of the AZORMAN study. Preliminary results from

this study are presented in **chapter 9**. **Chapter 4** and **5** describe different step-down treatment options for patients infected with an azole-resistant *A. fumigatus* strain when treated successfully with daily liposomal-amphotericin B. **Chapter 6** describes how azole-resistant Aspergillus CNS infections may be managed. **Chapter 7** shows the performance of a novel CE-marked point-of-care test: a lateral flow device. **Chapter 8** presents how azole-susceptible and azole-resistant *Aspergillus* co-infection can be diagnosed using *Aspergillus* qPCR test. The incidence and other characteristics of influenza-associated aspergillosis can be found in **chapter 10**. Future work is the subject of **chapter 11:** i. e. the protocol of the DUET study (azole-echinocandin combination therapy for IA). We conclude with a general discussion in **chapter 12**.

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