General summary and discussion

"Trust me, I know what I'm doing" (Sledge Hammer)

INTRODUCTION

An invasive fungal disease (IFD) is a life-threatening infection that is almost exclusively diagnosed in immunocompromised hosts. The most common invasive mould infection is caused by *Aspergillus* species and is called invasive aspergillosis (IA). Patients with acute myeloid leukaemia who are treated with intensive chemotherapy and haematopoietic stem cell transplant recipients are at highest risk for IA. Incidence rates of IA vary substantially and depend on host and environmental factors but also on the modalities of allogeneic stem cell transplantation recipients as well as the use of antifungal prophylaxis. 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 substantially higher medical costs [4]. The case fatality rate of IA is estimated to lie between 20-38% at 6 to 12 weeks after diagnosis, although considerable variation in incidence rates has been reported between populations [5]. Therefore, optimizing the management of IA is key 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. Nevertheless, the overall 6-week mortality is still unacceptably high at 25-30% even under treatment with voriconazole, combined with improved diagnostic tests [6]. A troublesome emerging problem in patients with IA is the increasing incidence of infections with triazole-resistant *A. fumigatus*. Although limited in numbers, case series have demonstrated that the overall mortality of patients infected with triazole-resistant *A. fumigatus* is very high (50-88%) [7, 8]. This thesis focuses on risk factors for and the diagnosis of invasive aspergillosis. Additionally, the management of azole-resistant aspergillosis is addressed. Below, I discuss the main findings of this thesis and conclude with the future perspectives that I envision.

DIAGNOSIS OF INVASIVE ASPERGILLOSIS: THE MATTER OF A DREAM TEAM

Consensus definitions

When a diagnosis of IA is made, the strength of the diagnosis is often reported according to the revised definitions of the European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) [9]. As such, IA is categorized into proven, probable and possible IFD. A proven diagnosis requires histopathologic evidence of fungal invasion or a positive culture from a sterile body site or fluid (e.g. pleural fluid

or CSF). 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/or with negative mycological criteria [10]. To fulfil mycological criteria, a positive direct test or indirect test is required. Direct mycological tests are the detection of typical fungal elements (e.g. septate hyphae with a 45° angle) or a culture positive for *Aspergillus species*. Indirect tests involve the detection of fungal antigens or cell wall constituents such as galactomannan antigen (GM) or beta-D-glucan [11]. Despite the fact that polymerase chain reaction (PCR) for the detection of *Aspergillus* in human specimens had been described more than two decades ago, the technique was not included in the 2008 EORTC/MSG consensus definitions due to the lack of standardisation [12]. Therefore, in 2006 the European Aspergillus initiative was founded (EAPCRI) to support an international platform for international standardisation. This has led to the incorporation of PCR in the most recent 2019 EORTC/MSG consensus definitions for diagnosing IFD [13]. Systematic reviews have concluded that *Aspergillus* PCR methods on BAL and blood provide a robust diagnostic test for the diagnosis of IA. An important and crucial step towards standardisation involves the use of a commercially available PCR like the aforementioned AsperGenius© quantitative PCR (qPCR). Although the clinical usefulness of this PCR is likely given the results of a retrospective study, large prospective multicentre studies on the real-life added value of this test are lacking [8, 14]. The Azole-Resistance Management study is such a study and is described later in this discussion and in **Chapter 9**.

Lateral Flow Device:

Galactomannan antigen detection and detection of *Aspergillus* DNA are labour intensive diagnostic tests with a turnaround time of at least 24 but typically 72 hours as they are mostly performed in batches in 96-well plates once or twice weekly. A timely diagnosis of IA is essential and improves clinical outcome, highlighting the need of a simple and rapid *Aspergillus* test that does not need to be performed in batches and that can be performed at any time of the day, also in small microbiology labs [15]. A newly CE-marked later flow device (LFD) might be the first of such tests. 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 [16]. We assessed the performance of this CE-approved LFD in a large multicenter retrospective study on a cohort of haematology patients from four large haematology centres in Belgium and The Netherlands [17]. These patients had undergone a diagnostic bronchoscopy with bronchoalveolar lavage fluid (BAL) sampling **(chapter 7).** The study included 247 patients of whom 79 had a proven or probable IA following the EORTC/MSG criteria [18]. In the primary analysis, only EORTC/MSG

proven cases were considered as true positives and patients with BAL samples that were culture and galactomannan negative, served as negative controls. The LFD showed a good diagnostic performance in this patient population known to be at high risk for IA. The sensitivity and specificity were 0.82 and 0.86 for visual readout and 0.82 and 0.96 respectively when a digital reader was used for the readout [17]. The LFD also showed an excellent negative predictive value of 0.98. However, the results should be interpreted with caution as proven cases are relatively rare and as always, the predictive value may differ substantially in populations with a different prevalence of IA. The EORTC/MSG criteria were used as a diagnostic reference but these criteria are subject to misclassification and incorporation bias (BAL galactomannan is one of the mycological criteria for probable disease). Therefore, the performance of LFD was also evaluated using the EORTC/MSG definitions with exclusion of galactomannan as mycological criterion. In this evaluation, LFD has similar sensitivity compared to galactomannan (0.76 versus 0.85, p=0.18) but was less specific (0.86 versus 0.96, p=0.005). This device can be used to diagnose but most importantly to exclude the disease with a high negative predictive value. This test can help in reducing the time to diagnosis of IA but will not replace GM, PCR or B,D-Glucan. First, it cannot be seen as an actual point-of-care test because hemorrhagic or viscous samples still need pretreatment with heating and the use of an EDTA-containing buffer. Second, in patients with a high pre-test probability, its sensitivity is too low to be used as a single diagnostic test. Nevertheless, we believe that this test has considerable value in combination with other indirect tests. IA can be excluded with almost 100% certainty when a BAL sample from a patient with a high pre-test probability for IA is triple negative (GM, PCR and LFD). In patients with a low pre-test probability, the use of 1 or 2 tests may suffice to rule out the diagnosis. As suggested by the title of this paragraph, the diagnosis of invasive aspergillosis is mostly made by circumstantial evidence and by combining different diagnostic tests. Therefore, a dream team is needed for the diagnosis of invasive aspergillosis.

Further prospective validation of the test is needed before more definite conclusions can be drawn. Very recently a second point-of-care test, the *Aspergillus* galactomannan lateral flow assay (LFA), was developed and CE-marked. It detects galactomannan and needs significantly less hands-on time in the lab to get to a result compared with the Platelia galactomannan test [19]. In a recent comparative multicentre study by Mercier *et al* [20], this LFA showed to be more sensitive and equally specific when compared to the LFD. Differences in sensitivity might be explained by the use of different targeted monoclonal antibodies and differences in pre-treatment steps in the lab as well as sample volume. Although the LFA has a higher sensitivity, the LFD is easier to perform because no pre-treatment steps are needed if the samples are non-viscous and not contaminated with blood.

AZOLE-RESISTANT ASPERGILLOSIS: TO WORRY OR NOT TO WORRY?

The Azole Resistance Management Study: past, present, future

IA is mostly, but not exclusively, caused by *Aspergillus fumigatus*. Azole-resistant *A. fumigatus* strains are an emerging global problem and significantly complicate the management of this infection [21]. Azole-resistance can develop in patients after prolonged treatment with azoles, primarily in patients with chronic pulmonary aspergillosis [22]. More importantly and more frequently, azole-resistance has an environmental origin and is the consequence of agricultural use of fungicides of the same azole drug class [22-24]. Therefore, the large majority of patients diagnosed with an azole-resistant *Aspergillus* infection have never received previous triazole therapy [25]. Azole-resistance is mostly caused by mutations in the *Cyp51A* gene that encodes for the lanosterol 14α-demethylase, the target enzyme for azoles. Two mutation combinations in this *Cyp51A* gene, the TR_{34}/L98H and the TR_{46}/T289A/Y121F pattern, account for more than 80% of the mutations conferring resistance in the Netherlands [26, 27]. The prevalence of azole-resistance rates vary substantially between geographic regions and between hospitals [21]. From a global perspective it is very remarkable that the highest prevalence of triazole resistance has been and continues to be documented in the Netherlands. It increased from 0% before the year 2000 to 5.3% in 2009, and further increased to a problematic prevalence of 15% in 2018 [7, 28]. In 2011, triazole resistance was observed in 5% of IA cases in Belgium as well. In 2017, researchers from the Erasme hospital in Brussels for the first time reported a prevalence rather similar to the Netherlands of 13% [29, 30]. Recently, it became apparent that this is not a unique problem limited to one hospital. Indeed, in 2019 the University Hospitals of Leuven, in which the largest number of patients with acute leukaemia is treated annually, described a prevalence of voriconazole resistance of 17% in their culture-positive IA cases at the department of haematology [31].

Detection of azole-resistant aspergillosis is challenging for several reasons. First, a positive fungal culture is required to allow for the use of conventional phenotypic resistance testing methods but in the majority of IA cases cultures remain negative. Second, phenotypic susceptibility testing according to internationally agreed methods is almost exclusively done in mycology reference labs and is time-consuming. Recently, the clinical usefulness and relevance of PCR-based testing for the presence of *Cyp51A* mutations was demonstrated in a study that used a now commercially available multiplex qPCR: i.e. the AsperGenius© qPCR [8, 14]. Besides detecting the presence of *Aspergillus* DNA, this qPCR allows for the detection of 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 a retrospective study showing a sensitivity and specificity of 89% and 89%, respectively, as compared with galactomannan and culture

results, which were used as the gold standard. In addition, this study showed that response to voriconazole therapy was poor, when given to patients infected with an azole-resistant *A. fumigatus* strain [8].

To obtain a reliable picture of the fungal infection management landscape in the Netherlands and in particular in the context of increasing triazole-resistance, we performed a survey questioning the prophylactic, diagnostic and therapeutic strategies regarding invasive fungal diseases in all academic Dutch haematology centres (**chapter 2**) [32]. Fungal prophylaxis during neutropenia was directed against *Candida* and in most centres consisted of fluconazole orally sometimes combined with oral amphotericin B suspension. Mould-active prophylaxis was given to acute myeloid leukaemia patients during chemotherapy in only 2 of the 8 centres. All centres used triazole prophylaxis in a subset of patients with graft-versus-host-disease. This survey showed that a uniform approach towards the diagnosis and in particular the treatment of invasive fungal disease in the context of an azole-resistance prevalence above 10% was lacking.

The results of the survey were processed, discussed and resulted in a protocol for a prospective multicentre study on the management of invasive fungal disease in haematology patients (The AZOle Resistance MANagement study (AzoRMan), NCT03121235). In this study, a standard diagnostic and therapeutic protocol for IA was agreed upon to be used as a guideline 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 study aims to demonstrate that the use of resistance testing by real-time PCR on BAL fluid from haematology patients with suspected IA will lead to a more rational and evidence-based management and an improved outcome for patients infected with an azole-resistant *A. fumigatus*. The use of PCR-based resistance testing is faster than culture-based methods and is more sensitive. Therefore, an earlier switch to appropriate non-azole therapy as soon as resistance is detected has become possible, hence potentially improving outcome. In addition, the AzoRMan-study aims to monitor the prevalence of IA due to *A. fumigatus* strains carrying the TR_{34}/L98H and TR_{46}/T289A/Y121F resistance-associated mutations 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 overestimation of the prevalence. Furthermore, the resistance rates that are now mostly reported are the result of a national surveillance program in which all cultures that are sent for antifungal susceptibility testing to the lab are used as denominator and the number of resistant cultures as numerator. The clinical relevance of these cultures is not always apparent, as it is a mixture of patients colonized with *Aspergillus species* rather than infected. (e.g. patients with structurally destroyed lungs, chronic pulmonary aspergillosis) and patients with invasive disease (ICU patients, patients with a haematological malignancy, solid organ transplant recipients, etc.). The overall incidence of azole-

resistance in the entire population of patients diagnosed with IA is therefore not entirely clear. This multicentre prospective study started in 2017 and is currently running in 11 haematology centres in the Netherlands and Belgium. In **chapter 9** preliminary results from the AzoRMan study are presented. To the best our knowledge, this is the largest prospective study evaluating the value of real-time PCR diagnosis of azole-resistance. As of December 2019, 212 patients have been included in the study. Galactomannan was positive (optical density of 1.0 or higher) on BAL fluid in 24% of the patients with available GM result. The AsperGenius® species and fumigatus PCR was positive in 40% and 29% of the patients, respectively. These numbers show that the majority of the patients with a haematological disease that undergo BAL sampling to confirm or rule out an IA, do not have this infection. Remarkably, in patients with a negative galactomannan on BAL, the *Aspergillus* species PCR was successful in 28% of patients. This shows that the best way to diagnose IA lies in the combination of different diagnostic assays, otherwise cases would have been missed. Real-time daily use of this qPCR facilitates the clinician in managing patients that otherwise would have been classified as possible IA.

At a recent international consensus meeting it was concluded that in geographical regions with a prevalence of triazole resistance of at least 10%, a switch from triazole monotherapy to L-AmB, or triazole and echinocandin should be strongly considered. Furthermore, every patient is at risk for azole-resistant aspergillosis in these regions because it is not the use of triazoles as prophylaxis or treatment but the inhalation of conidia from environmental *Aspergillus fumigatus* that became resistant through the exposure to triazoles in agriculture [33].

In chapter 3, we showed that inappropriate treatment of azole-resistant aspergillosis is associated with an increased overall mortality. A prevalence of azole resistance above 10% has been documented for several years in the Netherlands. Therefore, the Dutch guideline on the treatment of IA was changed in 2017. The guideline now recommends combination antifungal therapy (azole and echinocandin or azole and L-AmB) as one of the treatment options for patients suspected of having IA until resistance has been ruled out by culture or molecular diagnostic methods. In the 47 patients in whom the resistance PCR was successful in the AzoRMan-study, the prevalence of CYP51A gene mutations was 8.5%. Resistance seems lower than anticipated but data are too preliminary for definite conclusions to be drawn. Yet, treating all patients with non-azole antifungals like liposomal-amphotericin B comes at a cost of significantly more toxicity and higher costs. Furthermore, treatment duration often takes months. One may argue whether combination antifungal therapy is actually necessary with an observed resistance in fewer than 10% of patients. Furthermore, the vast majority of patients in the AzoRMan-study did not have IA. Starting combination antifungal therapy in all these patients would lead to an excessive use of non-azole antifungals. The AzoRMan study clearly supports another approach in this guideline. This approach consists of starting

azole-monotherapy while waiting for rapid antifungal resistance testing by PCR and culture, and streamlining therapy to the test results accordingly.

Resistance testing will not lead to an interpretable result in approximately 35-50% of the patients with IA (**chapter 9**). Indeed, fungal cultures remain negative in the majority of the patients with IA and PCR testing for *Cyp51A* resistance associated mutations is not always successful either. For this subgroup of patients, the SWAB guideline recommends to switch from triazole monotherapy to combination therapy as soon as it becomes clear that no resistance results will become available. The latter recommendation has been criticized when it relates to patients that are in good clinical condition, and have a lung-restricted, non-disseminated infection. Indeed, in my opinion, close monitoring for disease progression is a valid option because the poor outcome of azole-resistant IA has not (yet) been convincingly demonstrated for culture-negative cases if close radiological and clinical surveillance is done.

In only 47 of the 195 patients with available AsperGenius® PCR results, the resistance PCR was successful. Therefore, the sample size of the study population needs to be increased substantially in order to answer the primary research question. There remain some other urgent but open questions that cannot be answered at this point of time, but that will hopefully be answered when the AzoRMan-study will be fully enrolled. In particular, what is the outcome 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? How reliable is a negative resistance PCR result in culture negative but galactomannan positive patients?

Treatment modalities of azole-resistant aspergillosis

In the AzoRMan-study treatment with liposomal-amphotericin B (L-AmB) is advised when azole resistance is documented. This is supported by the fact that *A. fumigatus* strains are susceptible to L-AmB and is also advised by guidelines [33-35]. If a treatment response is observed during therapy with daily L-AmB 3 mg/kg, the study suggests two possible strategies of which the first is a switch to oral posaconazole in patients in which the posaconazole MIC of the *A. fumigatus* strain is below 2 mg/L and as long as posaconazole serum target trough level of 3-4mg/L can be achieved and tolerated. *Aspergillus species* carrying resistance-associated mutations often have MICs lower than 2 mg/L for posaconazole. *In vitro* and animal data suggest that they can be treated with posaconazole with therapeutic drug monitoring to ensure that high serum trough levels are obtained [36]. The efficacy of this strategy was demonstrated in a pharmacodynamic study in mice with azole-resistant IA. This study showed that posaconazole retains activity against an *A. fumigatus* strain with a posaconazole MIC of 0.5 mg/L as long as serum levels are sufficiently high. Human data on this strategy were only reported

anecdotally. Therefore, we describe in **chapter 4** the experience with the use of oral high-dose posaconazole as a treatment strategy in patients from two university hospitals in the Netherlands who were infected with moulds with a posaconazole MIC close to the clinical breakpoint. In the study, sixteen patients were intentionally treated with high-dose posaconazole. Grade 3-4 adverse events (AE) were observed in 6 patients and all of them were considered at least possibly related. Furthermore, we describe the adverse events observed in 25 patients with posaconazole concentrations at the higher end of the population distribution during treatment with the conventional licensed posaconazole dose. In this group of patients with spontaneously high posaconazole serum trough levels, grade 3-4 adverse events were observed in 5 of the 25 patients that were considered at least possibly related. The frequency of these side effects may be compared to intravenous treatment with L-AmB, which is associated with significant side effects as well. Therefore, we consider high-dose posaconazole a valid treatment option if strict monitoring for both exposure and adverse events (ECG for QTc time, electrolyte, liver enzyme and blood pressure monitoring) is possible.

The second strategy that we suggest in the AzoRMan-study is a step-down from daily L-AmB at 3 mg/kg to intermittent dosing of L-AmB 5 mg/kg three times a week. The long terminal half-life of L-AmB suggests that intermittent dosing could be effective, and can make outpatient antifungal therapy (OPAT) possible. L-AmB has a relatively short elimination half-life of 7 hours shortly after initiation of therapy, which increases to over 100 hours after prolonged use [37]. In **chapter 5**, we report our experience with the use of OPAT for IFD. All adult patients treated with L-AmB at a two- or three-times weekly administration frequency via the outpatient departments of four academic tertiary care centres in the Netherlands and Belgium in a time frame of 8 years were included in a retrospective cohort study [38]. In total, 18 patients were included and in 10 patients (66%) azole-resistant IA was the indication. The most frequently used regimen (67%) was 5 mg/kg 3 times weekly. In 94% of the patients a partial response to the daily treatment was confirmed by CT-scan before a switch from daily to intermittent dosing of L-AmB was made. An overall favourable outcome was achieved in 13 (72%) patients. The most important side effect was a decrease in renal function occurring in 10 (56%) cases. This was reversible in all and was treatment limiting in only one patient. 100-day mortality and 1-year mortality after initiation of OPAT were 0% and 6%, respectively. In a selected population like patients with azole-resistant IA, and after confirmation of initial response to treatment, our data support the use of outpatient antifungal therapy (OPAT) with L-AmB for treatment of IFD in a 3 times weekly dosing scheme. This treatment regimen of OPAT allows for a significant reduction in hospitalization duration and will therefore improve the patient's quality of life and the societal costs of treatment. A possible caveat of the favourable results that we observed for posaconazole and OPAT L-AmB might be patient selection. On the other hand, it illustrates that the decision

to choose one of these strategies that was made by clinician was done appropriately and probably in patients with a relatively favourable prognosis with regard to their IFD. Also, the heterogeneity of both the patient population and the different dosing regimens that were used for L-AmB makes it difficult to draw any definite conclusions about dosing, efficacy and tolerability. It is at the discretion of the physician to make a decision to apply these treatment options balancing the advantages of oral treatment and outpatient management versus the disadvantages described above. There are no validated other treatment options for azole-resistant aspergillosis as step-down therapy for daily L-AmB administration. Therefore, these case series are a welcome set of data to guide clinicians tackling these difficult-to-treat mould infections.

Outcome of azole-resistant aspergillosis

Case series indicate that IA caused by azole-resistant *Aspergillus* is associated with very high mortality rates of 50-88% [7, 8]. Until now, case series have included very few patients and preclude a reliable estimation of the impact of azole-resistance on mortality. Therefore, together with colleagues from Radboud UMC and Leiden UMC we performed a 5-year retrospective cohort study in order to compare the mortality between patients diagnosed with a voriconazole-susceptible and a voriconazole-resistant IA from 2011 to 2015. This study is described in **chapter 3** [39]. The clinical files of patients from which an *Aspergillus fumigatus* was cultured were investigated to identify patients with proven, probable and putative IA using the relevant classification definitions known as the EORTC/MSG or *AspICU* criteria [9, 10]. 196 patients with IA were eventually identified of which more than half had a haematological malignancy as the underlying disease. 37 of them (19%) harboured a voriconazole-resistant *Aspergillus fumigatus* strain. Mortality was higher in patients infected with a resistant compared to those with a voriconazole-susceptible strain: It was 21% and 25% higher at day 42 and 90 after the start of antifungal therapy, respectively. Patients that were not admitted to the ICU at the time of diagnosis had a 19% lower overall survival at day 42 when voriconazole-resistance was documented. In this study, antifungal therapy was considered appropriate if voriconazole was started in patients with voriconazole-susceptible disease and inappropriate in those with voriconazole-resistant IA. Thirty patients with voriconazole-resistant IA inappropriately received initial therapy with voriconazole at the time of first diagnosis of the IA. Therapy was switched to appropriate therapy (L-AmB) at a median of 10 days which illustrates the limitations of culture based resistance testing. Inappropriate initial therapy corresponded with reduced survival at day 42 compared with appropriate therapy (53 and 76%, respectively). One may argue that culture-positive IA cases have a higher fungal burden compared to culture-negative cases and will therefore have a higher mortality. Furthermore, resistance rates can be different in culture-negative cases with IA. However, in a single-centre study, resistance

prevalence was studied using culture-based strategy and using PCR. No difference was found in resistance prevalence using both strategies (11.7% versus 10.5%) [30].

Societal shortcomings and new kids on the block

While for good reasons a lot of attention, time and money has gone and continues to go to antibiotic stewardship programs, the problem of azole-resistance received much less attention. Even in the Netherlands, the global hot-spot of azole-resistant *A. fumigatus*, the national institute for public health and environment (RIVM) has not taken a nationwide initiative so far in order to map the epidemiology of azole-resistance in the Netherlands, let alone to tackle the source of the problem, in particular agricultural azole use. Outside the Netherlands, the problem is even worse as in many countries no data on the prevalence of azole-resistant *Aspergillus* are available. In the United States, the majority of the infectious diseases physicians are not familiar with the concept of azole-resistant aspergillosis and susceptibility testing is far from current practice [40]. As azole resistance is driven by agricultural use of fungicides, it is high time that strategies are developed to at least stop but preferentially reverse the continuous increase in prevalence of azole resistance. While the Netherlands is in the position to take the initiative to reduce the agricultural use of antifungals that are also used for the treatment of human disease, European cooperation is most probably needed. Meanwhile, new antifungal agents are being developed and studied in phase I and II clinical trials. However, these agents will not become available within the next several years, and will be extremely expensive. Furthermore, they may only offer a temporary solution without legislation regarding their non-use in agriculture. One of the compounds of which the clinical evaluation has been proceeding steadily is F901318 that was recently given the name olorofim. This synthetic small molecule inhibits dihydroorotate dehydrogenase (DHOH), which catalyses the conversion of dihydroorotate to orotate in the pyrimidine biosynthesis pathway [41]. Given its different mode of action from azoles, it is also active against azole-resistant *Aspergillus species*. It is currently being tested in a worldwide phase II trial [42, 43].

CNS azole-resistant aspergillosis

The most devastating form of IA is observed when the infection disseminates to the brain. Brain infections with *Aspergillus* have an extremely high mortality and all but few survivors are left with at least some neurological deficit [44]. Although the chances of survival have improved since voriconazole became available, the increasing prevalence of voriconazole resistance adversely impacts survival. Very few cases of central nervous system (CNS) aspergillosis caused by azole-resistant *Aspergillus fumigatus* have been reported, and most had a fatal outcome [33]. These 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, off-label use and/or uncommon routes of administration of antifungal agents may improve outcome. However, as cerebral infections with azole-resistant *Aspergillus fumigatus* are rare, large prospective studies are very difficult to perform. In **chapter 6,** we describe our experience with the use of intraventricular liposomal-amphotericin B (L-AmB) on top of systemic antifungal therapy in 3 patients**.** The patients were treated with L-AmB 1 mg given via a ventricular drain or reservoir on a weekly basis. Based on a theoretical total CSF volume of approximately 100-150 mL, the administration of 1 mg of L-AmB would result in a peak CSF concentration of L-AmB of 10 µg/mL. In a recent publication, the use of intrathecal or intraventricular L-AmB at a higher dose (10 mg daily for seven consecutive days) was shown to be well tolerated in 18 patients with cryptococcal meningitis [45]. A weekly administration of 1 mg L-AmB may not be optimal given this recent observation, and given the clearance of L-AmB is substantial because 500 mL of CSF is produced and reabsorbed each day. We therefore suggest that a higher dose as well as a more frequent administration should be strongly considered for future patients with azole-resistant cerebral IA. Measuring liquor levels of L-AmB may guide dosing. Therefore, a dose of 5 mg twice weekly may be suggested for these patients. Case series, as described in **chapter 6** have several limitations. In particular, all 3 patients received systemic treatment as well. In particular, the exact contribution of the intraventricular L-AmB administration is unclear. However, it is impossible that large prospective clinical studies will ever be performed. Therefore, treatment should be based on both preclinical data and thoroughly evaluated case reports.

Mixed infections

Mixed infections with azole-susceptible and azole-resistant strains of *A. fumigatus* have occasionally been described [46]. Until now, these cases of mixed infections had been documented by the demonstration of two different *A. fumigatus* strains with two different antifungal susceptibility profiles with conventional culture based methods [46]. However, the majority of cases of IA lack a positive culture. In **chapter 8,** we describe three patients infected with an azole-susceptible and azole-resistant *A. fumigatus* and in whom, for the first time, a mixed infection was demonstrated by *cyp51A* PCR amplicon melting curve analysis using the AsperGenius© assay. In these patients, wild-type and mutant *cyp51A* DNA from *A. fumigatus* was detected. In one case the mixed infection could be documented by culture as well by showing growth of an azole-susceptible and azole-resistant strain. In the two other patients, the cultures remained negative. Without the application of a molecular assay (AsperGenius© PCR), these mixed infections would have been missed [47]. Different *Aspergillus* isolates can be present within the same host. One of the isolates can become dominant and disseminate causing disease.

This study demonstrates that even when an azole-susceptible strain is cultured, the patients can still harbour an azole-resistant *A. fumigatus* isolate. Therefore, in azole-resistant endemic regions we advocate that at least five and preferably all colonies that are cultured on the agar plate are phenotypically tested for the presence of azole-resistance. Importantly, molecular assays should always be used in combination with conventional susceptibility testing as they can only detect the mutations that are included in the assay and new mutations or resistance mechanisms may occur.

INFLUENZA-ASSOCIATED ASPERGILLOSIS: A NOVEL AND LETHAL UNDERESTIMATED ENTITY

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 [48, 49]. 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) remained 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. We performed a large retrospective multicentre cohort study of adult patients admitted to the ICU with severe influenza infection and acute respiratory failure. Data were collected in 7 ICUs across Belgium and The Netherlands. All patients had a confirmed influenza infection based on a positive airway PCR test. The aforementioned EORTC/MSG criteria are used to classify patients with a fungal infection in an immunocompromised host but are not applicable to the intensive care setting. Therefore, an algorithm (*AspICU*) was described by Blot *et al.* to distinguish invasive pulmonary aspergillosis from *Aspergillus* colonisation in patients who are critically ill [10]. However, the entry criterion for this algorithm is a positive culture of *Aspergillus* species and cannot be applied to determine the incidence of *Aspergillus* infection in this cohort of severe influenza patients because the majority of cases are culture-negative. We applied a modified definition of invasive aspergillosis using the *AspICU* algorithm and this definition was based on the presence of clinical, radiological, and mycological criteria (see **chapter 10.1** for the full definition) [50]. This definition did not require an EORTC/MSG-defined host factor because otherwise non-immunocompromised patients with severe influenza would never fulfil the definition. The influenza patient cohort consisted of 457 patients of which 25 patients with *Aspergillus* colonization of the airways were excluded. These 25 patients had a positive *Aspergillus* culture from a lower respiratory tract sample but had a negative or unavail-

able BAL culture or galactomannan test and were excluded because it was impossible to determine if these patients were colonized or had invasive disease. 83 of the remaining 432 patients (19%) fulfilled the modified IA definition. Mortality was higher in patients with influenza-associated aspergillosis when compared to patients without *Aspergillus* superinfection, 45% versus 20%, respectively. Remarkably, one out of three patients who were immunocompromised according to the EORTC/MSG criteria had an *Aspergillus* superinfection and 71% of them died within 90 days after ICU admission [9, 51]. Our results are in line with smaller retrospective studies that have reported similar rates of IAA superinfection [52, 53]. A recent study have reported lower rates of IAA [54]. These differences might be explained by different diagnostic strategies or tests that are used (e.g. the application of galactomannan testing on BAL fluid, local awareness of the problem of IAA, or differences in still to be elucidated geographical or host factors). Furthermore, our study was mostly conducted in tertiary referral centres that may have led to the inclusion of a sicker population with more severe respiratory failure that is necessarily captured by the APACHE II score at admission.

Besides a higher APACHE II score and male sex, the third independent risk factor for the occurrence of *Aspergillus* superinfection that we observed in patients admitted to the ICU with influenza was corticosteroid therapy in the 4 weeks preceding ICU admission. A recent Cochrane systematic review concluded that the administration of corticosteroids to patients with influenza admitted to the ICU is associated with higher mortality [55]. Our data are in agreement with this review and although a randomized study on the use of corticosteroids in patients with severe influenza is lacking, the available data seem to argue against its use. Another important observation was that the diagnosis of IA was made shortly after admission (median of 3 days). The data preceding our study suggested that almost all cases of IAA were diagnosed in patients infected with the pandemic influenza A H1N1. A recent single-centre case study reported that influenza B could trigger *Aspergillus* superinfection as well [56]. We observed that the incidence of IAA in patients admitted to the ICU with influenza B is comparable to patients admitted with influenza A (**chapter 10.1**) [50].

To determine whether invasive aspergillosis is independently associated with influenza, we included a control cohort of patients admitted to the ICU with severe community-acquired pneumonia (CAP) and respiratory insufficiency, similar to patients with influenza. We excluded immunocompromised patients from this analysis to focus on the risk of influenza and bacterial pneumonia per se as a risk factor. 45 patients (14%) of the 315 non-immunocompromised influenza cohort were diagnosed with *Aspergillus* superinfection compared to 16 (or 5%) of the 315 CAP patients in the control cohort. We performed a binary logistic regression analysis to assess whether influenza was independently associated with IA in the pooled cohort of non-immunocompromised influenza-positive and influenza-negative patients. This analysis showed that influenza

infection was independently associated with the development of invasive aspergillosis. By choosing patients with severe community-acquired pneumonia as a comparative group, we can only conclude that the presence of influenza is a risk factor for invasive aspergillosis compared with this control group. We considered this control group the most appropriate because, just like patients with influenza, respiratory failure was the primary reason of the ICU admission.

Little is known about the pathophysiology of influenza-associated aspergillosis. Respiratory epithelium damage and mucociliary clearance dysfunction might facilitate invasion of *Aspergillus* [57]. Another explanation might be that influenza induces immunoparesis and also induces cytokine release that negatively impacts the innate and adaptive immune response [57]. Another bold explanation for the increasing rates of IAA might be the use of oseltamivir, a neuraminidase blocker that is administered in patients with influenza. *In vitro* research has shown that neuraminidase activity is important for *Aspergillus* immune responses. Treatment with oseltamivir, thus blocking host neuraminidase activity, might therefore increase susceptibility for *Aspergillus* infection [58]. In our cohort, 90 patients did not receive a neuraminidase inhibitor and 13 (14.5%) patients had IAA in this cohort. 338 patients received a neuraminidase inhibitor and 70 patients had IAA in this cohort (21%). This trend towards an increased incidence was not statistically significant (p=0.18) and needs further study before any conclusions can be drawn.

We performed a mortality analysis on our influenza cohort of 432 patients admitted to the ICU with influenza to evaluate whether or not 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 (see **chapter 10.2**) [59]. We therefore performed a cox regression analysis showing that the emergence of IAA was independently associated with 90-day mortality. Although we acknowledge that observational data can never prove a causal relationship, the association of IAA and mortality was independent of confounders like severity of illness and being immunocompromised at ICU admission. This finding again confirms the relevance of diagnosing IAA in the ICU. In accordance with recent literature, corticosteroids exposition before ICU admission significantly impacted mortality as well, and strongly suggests that caution is needed regarding the use of adjuvant corticosteroid therapy for patients with severe pneumonia during the influenza season.

Our study clearly shows that invasive aspergillosis is a frequent and lethal complication in patients admitted to the ICU with influenza pneumonia. A large part of our patients with IAA cannot be classified using the current diagnostic criteria (EORTC/MSG and *AspICU*) [9, 51] because influenza is not considered a host factor in these criteria. In December 2019 updated EORTC/MSG consensus definitions of invasive fungal diseases were published [13]. Unfortunately, patients admitted to the ICU with influenza were

not included in the newly defined host factors. Therefore, it seems that the current definitions are already outdated in this regard. Application of these criteria would lead to missed diagnosis. Unfortunately, in Belgium the EORTC/MSG criteria are used for reimbursement of antifungal drugs, although these criteria were never meant to be used by a clinician, let alone to base reimbursement policies of drugs on. They were developed to design clinical trials uniformly. In addition, autopsy series have shown that strict interpretation of host criteria contributes to missed diagnosis of IA, in particular in the ICU [60]. Restricting reimbursement of antifungal drugs to EORTC/MSG defined cases of IA should therefore be abandoned.

INITIATED STUDIES AND FUTURE DIRECTIONS

Azole-echinocandin combination therapy for invasive aspergillosis

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 [61, 62]. 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 [63]. In this trial, 6-week mortality was 30% lower in the group treated with combination antifungal therapy (19.3%) versus monotherapy (27.5%) but this was not statistically significant (p=0.09). However, a difference in overall mortality of 30% would already be very important. This study had a 70% power for an unrealistic overall mortality decrease of 65% rather than 30%. Therefore, no conclusions can be drawn and combination therapy has not been adopted by current guidelines so far. In a post-hoc analysis of the 222 patients with radiographic abnormalities and a positive galactomannan antigen test, a statistically significant difference in mortality was observed (p=0.037). A second clinical trial is therefore needed to confirm these promising findings. In 2019, dr. B. Rijnders, drs. A. Schauwvlieghe and Prof. dr. J. Maertens submitted a study proposal to the first grant call by BeNeFit (Belgium-Netherlands Funding of International Trials) and 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. Given the evidence in favour of voriconazole-echinocandin combination therapy as well as the increasing incidence of voriconazole-resistant *A. fumigatus* in Belgium and the Netherlands, a large clinical study on the value of combination therapy is needed. Furthermore, this trial will allow for a reliable measurement of the incidence of azole-resistant IA in the Netherlands and Belgium continuing the main research aim of the AzoRMan-study (**chapter 9**).

The study is designed as a large pragmatic clinical trial to facilitate enrolment as much as possible. In particular, we want to leave the choice of the triazole (voriconazole or isavuconazole or posaconazole IV or oral) to the treating physician. This will not only lead to less patients being excluded but also allow the clinician to switch from one drug to another (within the same class) in case of treatment limiting toxicity. With the unbiased endpoint of overall 6 weeks mortality, we consider a pragmatic approach that allows for easy recruitment of a sufficient number of patients more important than the use of one specific drug within a class or the use of a placebo. Combination therapy will be discontinued after 28 days in all patients in which triazole susceptibility was documented but when a treatment response is observed before day 28, the echinocandin can be discontinued as from day 7. Phenotypic real-time resistance testing will be performed on side using the VIPcheck test while genotypic resistance testing will be done in reference labs in both countries with the use of the AsperGenius® PCR [8, 64].

Some patients will be excluded after randomization: patients in whom resistance is shown by PCR or culture, patients in the Netherlands in whom resistance cannot be excluded (culture and PCR not successful) and patients included at the time when the diagnosis of IA was possible but not probable or proven and in whom an upgrade to probable or proven IA is not achieved within 7 days after the start of antifungal therapy. These patients cannot be seen as collateral damage. Data will be collected of these patients and this study will give better insight in the outcome of patients with azole-resistant aspergillosis. In addition, information will be available on patients with documented azole-resistance IA when treatment is started with azole monotherapy or combination therapy. Patients that are excluded because resistance testing did not give a result, will deliver interesting information on the treatment of IA cases in which no information on azole-resistant aspergillosis is available. This DUET-trial will open in 2020 and will enroll patients in 25 centres in Belgium and The Netherlands and contacts are being made with centres in Scandinavia as well to allow for swift recruitment. Expected accrual time is 3 to 4 years. This study elegantly continues the work presented in this thesis by (1) measuring the incidence of azole-resistance in the lowlands, (2) hopefully improving the outcome of patients with IA and influenza and (3) evaluating the effect of combination antifungal therapy on outcome of azole-susceptible IA.

Azole-Resistant PCR Optimalization Study on serum study (ARPOS)

BAL sampling is invasive, costly, labour intensive and not always feasible in haematology patients. Therefore, the validation of the AsperGenius® assay to easily obtainable serum samples would be very advantageous. A small single centre study, showed that the AsperGenius® assay can detect *Aspergillus* DNA and azole resistance on DNA isolated from serum samples [65, 66]. However, successful amplification of regions associated with azole resistance from serum samples was achieved in only 50% of the patients. There-

fore, the diagnostic use of an azole resistance PCR shows promise but the sensitivity is clearly suboptimal when small serum volumes (0.5 or 1ml) are used. In 2008, Suarez *et al*. showed that DNA extraction from large serum volumes improved the diagnostic yield of a serum *Aspergillus* PCR [67]. DNA extraction is a critical process to the success of most PCR amplification systems [68]. Therefore, we think that the detection of *Aspergillus* DNA and resistance associated mutations on serum could be further enhanced by extracting DNA from relatively large serum sample volumes (3 or even 10 ml) and by using greater DNA template volumes (>10 µl). In 2017, we started a prospective study collecting large serum and plasma samples of patients with haematological disease on the day the patient undergoes BAL sampling to exclude invasive fungal disease. This study will therefore prospectively examine the performance of DNA extraction and PCR from large volume serum and plasma samples of patients with haematological disease suspect of having (resistant) IA. The results of the PCR performed on serum/plasma will be compared with the results obtained on BAL samples. The objective of the study is to determine the best medium for *Aspergillus* DNA extraction, to determine the best serum/plasma volume to generate the most sensitive and specific *Aspergillus* PCR results and to compare different (commercially) available *Aspergillus* species PCR's.

Upfront chest CT: a screening tool for IA in patients with acute leukaemia?

Diagnosis of IA is not only dependent on biomarkers but imaging is an essential part of the diagnostic steps towards a timely diagnosis of IA in patients with haematological malignancy. Recently, a prospective cohort study was published that evaluated the value of a baseline chest CT scan in high-risk haemato-oncological patients. In 107 patients with AML, a baseline CT scan was performed within days after the diagnosis of IA was established. In this cohort, 20 patients were diagnosed with proven or probable IA at any time during hospitalisation for induction chemotherapy. Remarkably, half of these cases were diagnosed at admission preceding the start of chemotherapy [69]. Another study by Ceesay and colleagues prospectively evaluated baseline chest CT among 198 high-risk haemato-oncologic patients and found that a pathological baseline chest CT and EORTC/MSG-compatible CT findings was associated with a hazard ratio of 2.52 (95% confidence interval [CI] 1.27–5.03) and 4.67 (95% CI 2.04–10.75), respectively, for subsequent diagnosis of IA. The median time to diagnosis of IA was 14 days. Yet, the studied patient population was heterogeneous, consisting mainly of heavily pre-treated patients [70]. The main concern of many clinicians for applying a baseline CT scan to all patients receiving induction chemotherapy is that non-clinically relevant findings will lead to a substantial amount of unneeded diagnostic testing. This was confirmed by a retrospective cohort study. This study reported that about two thirds of patients with AML had atypical lesions on baseline chest CT performed before or on the day of induction chemotherapy initiation [71]. This might lead to many unnecessary bronchoscopies

being performed in patients that already have much to endure at the time of AML diagnosis. At Ghent University Hospital baseline chest CT is already common practice since 2012 in newly diagnosed AML patients. We are performing a retrospective cohort study to evaluate the value of baseline chest CT at admission.

DB-MSG: new consortium for future research

In 2017 the Dutch-Belgian mycoses study group (DB-MSG) was founded following the many multicentre projects that have been performed in the past (www.DBMSG.nl). This study group allows easy networking and enables a closer partnership between Belgian and Dutch academic centres in order to tackle invasive fungal disease together. A large volume of precious biological material of blood and BAL samples is and will be available with the past and future studies performed by the DB-MSG (AzoRMan, ARPOS, DUET study as well as the PosaFlu studies that are and will be performed) allowing the validation of existing and new biomarkers. A possible biomarker could be new cytokines like IL-6 and IL-8. A recent study has shown that elevated levels of IL-6 and an IL-8 in blood and BAL fluid at the time of bronchoscopy and rising levels in blood 4 days following bronchoscopy were predictive for mortality in patient with haematological malignancy undergoing bronchoscopy for suspected IFD [72]. Cytokines are involved in the protective immunity against *Aspergillus species* and might facilitate treatment stratification and may function as surrogate markers for disease status in the future. Another marker that could be studied in this cohort is the use of mass spectrometry to measure panfungal serum disaccharide [73]. The prospective validation of the usefulness of new lateral flow devices is another study that should be performed, in particular in patients other than those with haematological disease.

Many centres apply antimould prophylaxis for AML patients and for patients receiving an allogeneic stem cell transplant. Several upcoming anti-leukemia drugs that specifically target pathogenic mutations will be combined with the classic intensive chemotherapy regimen (3+7) for the treatment of patients with AML. This will make the universal administration of azole prophylaxis to AML patients in many haematology units challenging. Indeed, due to the fact that most of these targeted therapies are metabolized by CYP enzymes, caution should be taken regarding azole induced CYP450 enzyme inhibition. The opinions about the value of a good diagnostic-driven approach compared with the use of universal azole antimould prophylaxis differ substantially. However, the superiority of one of both strategies has never been demonstrated conclusively. An ideal approach would be to have a more individualized approach by detecting patients with the highest risk for an IFD. A possible approach would be by the detection of single nucleotide polymorphisms (SNPs) that are associated with an increased risk to develop IFD. By the detection of a SNP, a possible selection could be made for patients that are at highest risk and in whom anti-mould prophylaxis can be expected to be most

valuable. A possible SNP, that could help to detect these patients, is pentraxin 3 (PTX3) deficiency. A randomized trial is needed to demonstrate that the detection of these risk-markers can benefit patients.

CONCLUSION

Several studies were performed to improve our knowledge on the incidence, mortality, risk factors and diagnosis of IA. In chapter 3, we demonstrate that culture-positive azole-resistant IA is associated with a higher overall mortality. In chapter 2, we described that in hospitals in the Netherlands in the context of an ever-increasing prevalence of azole-resistance the management of IA is diverse. With the AzoRMan-study, described in chapter 9, we implemented a uniform diagnostic-driven approach towards patients with a suspected IA. This study in 11 hospitals will not only result in a better overall picture of the prevalence of triazole resistance in this patient population but also demonstrate the exact value of PCR-based resistance testing on BAL fluid of haematology patients. Preliminary results from this study show that the majority of patients with haematological disease that undergo broncho-alveolar lavage sampling do not have invasive aspergillosis. In the patients in whom the resistance PCR was successful, the prevalence of Cyp51A gene mutations was 8.5%. The sample size has to be increased substantially to answer the primary research question because only in 25% of patients the resistance PCR led to an interpretable result. In chapter 4 and 5, we describe our experience with different step-down treatment options for patients infected with an azole-resistant *A. fumigatus* after initial induction therapy with daily liposomal-amphotericin B. We showed that posaconazole can be used while targeting higher than normal serum levels as long as appropriate safety measures are taken into account. Another valuable option is the use of intravenous L-AmB as outpatient antifungal therapy when administered three times weekly. In chapter 6, we describe our experience with the use of intraventricular L-AmB as a last resort therapy for patients with cerebral IA. In chapter 7, we demonstrated that a CE-marked lateral flow device that was developed to be used as a rapid diagnostic test for IA, performed well compared with the current gold-standard. In chapter 8, we showed that mixed infections with azole-susceptible and azole-resistant *Aspergillus fumigatus* can be diagnosed with the AsperGenius© PCR. Finally, in chapter 10, we demonstrated that in patients admitted for respiratory insufficiency, an infection with influenza is an important risk factor for the development of IA. We also showed that IAA was independently associated with a higher mortality. Several new studies are enrolling patients or will do so in the near future (ARPOS, DUET studies). We hope that they will improve the management and eventually outcome of patients with an invasive fungal infection.

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