RISK FACTORS, DIAGNOSIS AND MANAGEMENT OF (AZOLE-RESISTANT) INVASIVE ASPERGILLOSIS

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Risicofactoren, diagnose en behandeling van (azole-resistente) invasieve aspergillose

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Ter nagedachtenis van mijn lieve Moetie

Voor mijn lieve vrouw Judith,
mijn zoontje Oscar
en ons toekomstige dochtertje

Chapter 1

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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Chapter 2

The diagnosis and treatment of invasive aspergillosis in Dutch haematology units facing a rapidly increasing prevalence of azole resistance. A nationwide survey and rationale for the DB-MSG 002 study protocol.

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Education never ends, Watson. It is a series of lessons, with the greatest for the last. (Arthur C Doyle)

SUMMARY

Background

Patients with haematological malignancies are at risk for invasive fungal diseases (IFD). A survey was conducted in all Dutch academic haematology centres on their current diagnostic, prophylactic and therapeutic approach towards IFD in the context of azole resistance.

Methods

In all 8 centres, a haematologist and microbiologist filled in the questionnaire that focused on different subgroups of haematology patients.

Results

Fungal prophylaxis during neutropenia was directed against *Candida* and consisted of fluconazole and/or amphotericin B suspension. Mould-active prophylaxis was given to acute myeloid leukaemia patients during chemotherapy in two of eight centres. All centres used azole prophylaxis in a subset of patients with graft-versus-host disease. A uniform approach towards the diagnosis and treatment of IFD and in particular azole-resistant *Aspergillus fumigatus* was lacking. In 2017, all centres agreed to implement a uniform diagnostic and treatment algorithm regarding invasive aspergillosis with a central role for comprehensive diagnostics and PCR-based detection of azole resistance. This study (DB-MSG 002) will re-evaluate this algorithm when 280 patients have been treated.

Discussion

A heterogeneous approach towards antifungal prophylaxis, diagnosis and treatment was apparent in the Netherlands. Facing triazole-resistance, consensus was reached on the implementation of a uniform diagnostic approach in all eight centres.

INTRODUCTION

Invasive fungal disease (IFD) occur in 5 to 40% of patients with haematological malignancies. Approximately 95% of the IFD are caused by *Aspergillus* and *Candida* species.[1] IFD is associated with a very significant morbidity and mortality that is explained by the difficulties in diagnosing IFD rapidly.[1] In addition, the presence of an IFD leads to a delay in subsequent anti-leukemic therapy, and therefore also indirectly affects the outcome of the patient.[2]

Antifungal prophylaxis prevent IFD during acute myeloid leukaemia (AML) therapy or during graft-versus-host disease (GVHD). These benefits have to be weighed against risks of drug toxicity, interactions, selection of resistance and costs. Different opinions on the preferred antifungal strategy in these patients exist and the approach varies considerably from institution to institution.

Over the last 10 years resistance of *A. fumigatus* against triazoles, has become a significant problem in the Netherlands but has recently also been reported in other countries.[3-5] Triazole-resistance can develop through long-term azole therapy in patients with chronic pulmonary aspergillosis. However, the selection of tri-azole resistance in the environment by the use of azole fungicides is far more important. This in agreement with the observation that the majority of triazole-resistant *A. fumigatus* strains contain the environmental TR_{34}/L98H or the TR_{46}/Y121F/T289A mutation pattern in their *Cyp51A* gene.[6] This gene encodes for the target enzyme of triazoles.[7] Infections with a triazole-resistant *A. fumigatus* result in a high mortality and the best diagnostic and treatment approach is uncertain.[5, 8] We conducted a survey on fungal diagnostics, antifungal prophylaxis and treatment in all Dutch academic haematology centres. The survey facilitated the development of a consensus approach towards the management of invasive aspergillosis (IA) in a context of rising azole resistance.

Materials and methods

A questionnaire was sent to a haematologist and a microbiologist with special interest in supportive care and medical mycology respectively and both parties were asked to answer as a team for their centre. The questionnaire focused on (1) primary prophylaxis during AML chemotherapy, during allogeneic hematopoietic stem cell transplantation (allo-HSCT) and at the time of GVHD. (2) How was screened for IFD and which diagnostic tests were performed. (3) The current antifungal treatment for different clinical scenarios. The results were processed and during a consensus meeting the protocol for the AzoRMan-study was developed and implemented.

RESULTS

Prophylaxis (table 1)

Prophylaxis directed against Candida

Fluconazole is given during neutropenia of >10 days in 4/8 centres at very different dosages and amphotericin B oral suspension was used in 2. One centre also uses amphotericin B lozenge. One centre starts fluconazole when surveillance cultures grow *Candida*. If surveillance cultures show *Candida* species resistant to fluconazole, some centres switch to amphotericin B suspension and one centre adds amphotericin B suspension to fluconazole. Finally, one centre stops fluconazole and no other prophylaxis is initiated.

Mould-active prophylaxis

Only one centre applies mould-active prophylaxis (itraconazole) during chemotherapy induced neutropenia of >10 days and during myeloablative allo-HSCT. Therapeutic drug monitoring of itraconazole is performed and when no effective plasma concentrations are reached, a switch to voriconazole is made. In another centre nebulized liposomal amphotericin B (L-AmB) at 15mg QD, twice weekly is used for this purpose. All centres start mould-active prophylaxis when corticosteroids are given for GVHD but the drugs of choice differ (table 1).

	Antifungal agent	Dosage	Number of centres
***Candida* prophylaxis during longstanding chemotherapy-induced neutropenia**	Fluconazole	50mg /24h	1
		200mg /24h	2
		400mg /24h	1
	Amphotericin B suspension	500mg/6h	2
		200mg/12h	1
	Fluconazole when surveillance cultures grow *Candida*		1
Anti-mould prophylaxis in AML/MDS/AlloTx during longstanding chemotherapy-induced neutropenia	Itraconazole suspension	Start with 200 mg bid, dose increased based on TDM results	1
	L-AmB aerosols	15mg twice weekly	1
	None		6
AlloTx with GVHD treated with systemic corticosteroids	Itraconazole	Start with 200 mg bid, dose increased based on TDM results	1
		2,5mg/kg/12h	1
	Voriconazole	200mg/12h	1
	Posaconazole	300mg/24h tablets	5

Table 1: Prophylactic strategies used against *Candida* and *Aspergillus*.
AlloTx=Allogeneic stem cell transplantation; AML=Acute Myeloid Leukaemia; GVHD= Graft-versus-Host disease; MDS=Myelodysplastic syndrome; TDM=Therapeutic Drug Monitoring. L-AmB=liposomal amphotericin-B.

Diagnosis

Diagnostic procedures (table 2)

A chest CT is routinely performed in all centres after three to five days of neutropenic fever without an infectious focus despite antibiotic therapy. When the chest CT scan shows pulmonary infiltrates a broncho-alveolar lavage (BAL) with galactomannan (GM) detection and fungal culture is performed in all centres (if clinically feasible). Twice weekly serum GM monitoring as a screening tool is performed in one centre only. Two centres perform an *Aspergillus* DNA PCR on BAL routinely; in one centre this is done only when BAL GM is positive or when an EORTC compatible radiological finding is suggestive of an IFD.

Diagnostic procedure	Possibilities	Nr of centres
Screening with serum GM (twice weekly) during prolonged neutropenia	Yes	1
	No	7
Chest CT-scan when 3-5 days neutropenic FUO despite broad-spectrum antibiotic treatment	Yes	8
	No	0
Bronchoscopy with BAL (when no evident cause for infiltrative lesions on imaging)	Yes	8
	No	0
GM measurement on BAL fluid sample, if BAL sampling is performed	Yes	8
	No	0
***Aspergillus* species PCR on BAL fluid**	Yes, always	2
	Yes, if GM is positive	1
	No	5

Table 2: Diagnostic strategies used in patients at risk for or suspected of having an IFD. BAL=Bronchoalveolar lavage; GM=Galactomannan; FUO=Fever of unknown origin. IFD=Invasive Fungal Diseases

Susceptibility testing (table 3)

Different Aspergillus susceptibility testing methods are used: VIPcheck™ or Etest followed by confirmation with testing according to the European Committee on Antibiotic Susceptibility Testing (EUCAST) method when resistance is suspected based on the screening assay. The EUCAST method is operational in the mycology reference laboratory (RefLab). Resistance screening is done in all but one centre with a 4-well plate (VIPcheck™) in which three of the four wells contain agar supplemented with an azole (voriconazole, itraconazole and posaconazole) and the fourth functions as a growth control. The other centre uses the Etest (bioMérieux) for resistance screening. Simultaneously to the screening test, four centres send the *Aspergillus* strain directly to the RefLab for MIC testing. PCR testing for the presence of TR_{34} and TR_{46} directly on cultured *A. fumigatus* colonies is performed on-site in four centres to speed up resistance detection. A PCR-based resistance assay is performed directly on BAL in 3 centres. For this purpose, a commercially available qPCR (AsperGenius®) or an in-house PCR is used. One centre sends BAL samples to the RefLab for PCR testing.

Susceptibility assay	Possibilities	Nr of centers
***Aspergillus species*: Screening for azole resistance with VIP™check-testing**	Yes	7
	No	1 Sends *Aspergillus* strain directly to RefLab
Phenotypic azole resistance testing (EUCAST) of cultured *Aspergillus* strains	Directly sent to RefLab for EUCAST testing	4
	Send to RefLab only if VIP screening is positive	2
	Send to RefLab only if E-test is positive	1
	EUCAST testing on site=RefLab	1
Testing for RAM on cultured *Aspergillus* strains	Yes, in-house	4
	Yes, not in-house	1
	No	3
Testing for RAM (*CYP51A*) directly on BAL fluid	Yes	2
	No	4
	On indication (if BAL culture is negative and patient is not doing clinically well)	1
	Sends BAL sample to the RefLab	1

Table 3: Diagnostic tests done on BAL fluid samples.
EUCAST=The European Committee on Antimicrobial Susceptibility Testing; GM=Galactomannan; VIPTM testing=resistance assay (explanation: see text); RAM=Resistance associated mutations (TR34/L98H, TR53, and TR46/Y121F/T289A); RefLab=National mycology reference laboratory in Nijmegen (The Netherlands). BAL=Broncho-alveolar lavage.

Treatment (table 4)

Suspected invasive fungal infection:

All centres use voriconazole as the initial treatment for patients in a respiratory stable condition suspected of having an IFD while waiting for the microbiological tests. One centre frequently uses posaconazole as well and another centre with a high local azole resistance prevalence prefers L-AmB if the patient is very ill. The feasibility of BAL fluid sampling is the decisive factor in another centre to guide therapy and voriconazole is given if a BAL is obtained and therefore, the detection of azole resistance becomes more likely. If BAL is not feasible, this centre gives L-AmB as antifungal.

Proven or probable IA:

Voriconazole is the treatment of choice for all centres when a BAL-GM assay is positive in a respiratory stable patient and the lesions on chest CT are not widespread, fungal culture remains negative and no susceptibility PCR is performed or the test was not successful. In the same clinical situation with a patient in respiratory distress or with extensive pulmonary infiltrates, five centres would still start voriconazole. Two centres would start L-AmB and one centre posaconazole.

Presentation	Clinical condition	Treatment options	Nr of centers
Chest CT: suspected IFD but microbiological results pending	Respiratory and clinically stable	Voriconazole	8
	Respiratory and clinically instable	Voriconazole	6
		L-AmB	1
	+BAL possible	Voriconazole	1
	+BAL impossible	L-AmB	
BAL GM pos, Culture/PCR neg	Respiratory and clinically stable	Voriconazole	8
	Critically ill	Voriconazole	5
		L-AmB	2
		Posaconazole	1
Resistance detected by culture or PCR	Respiratory and clinically stable/ instable	L-AmB	8
TDM voriconazole		No	2
		Sometimes*	2
		Always	4
TDM posaconazole		No	2
		Sometimes*	3
		Always	3

Table 4: Treatment of invasive aspergillosis
BAL=Bronchoalveolar lavage; Resp=Respiratory; L-AmB=liposomal amphotericin-B; IFD=Invasive Fungal Diseases; PCR=Polymerase Chain Reaction; GM=Galactomannan; TDM=Therapeutic Drug Monitoring; +BAL possible/impossible: BAL sampling was possible/impossible; *Sometimes=when toxicity or therapeutic failure is suspected

Proven or probable IA and documented voriconazole resistance

If voriconazole resistance is demonstrated with one of the phenotypic susceptibility tests or by a resistance PCR, all centres give L-AmB.

Therapeutic drug monitoring

Voriconazole

Two centres do not perform therapeutic drug monitoring (TDM). Two centres do TDM when toxicity or treatment failure is suspected. The other centres routinely perform TDM.

Posaconazole

Three centres always perform TDM and two centres do not. The other three centres perform TDM on indication only.

Triazole resistance data

In 2016 *A. fumigatus* isolates from 784 clinical patients were screened for triazole resistance using a four-wells agar plate (VIPcheck™). Isolates that grew on the triazole-

containing agar have a high probability of resistance and were sent to the Reflab for phenotypic and genotypic characterization. 101 isolates (12.9%) were triazole-resistant, which was higher than 2014 (7.2%) and 2015 (10.7%). In individual centres, resistance ranged from 9.5% to 20.5%.[6] Recently, a nationwide Dutch cohort study reported data from 144 patients with influenza pneumonia admitted to all eight University Intensive Care Units. 23 patients (16%) were diagnosed with influenza-associated invasive aspergillosis and triazole resistance was reported in 29% of those with a positive *A. fumigatus* culture.[9]The clinical relevance of triazole resistance was also described in another recent study in which a multiplex real-time PCR test (AsperGenius© assay) was performed on BAL samples from 201 patients. This qPCR allows the simultaneous detection of *Aspergillus* species and identification of the most common mutations in the *A. fumigatus Cyp51A* conferring resistance by using melting curve analysis. In 11 of the 68 patients in which the resistance PCR could be successfully performed, the TR_{34}/L98H or TR_{46}/T289A/Y121F resistance pattern was documented. More importantly, the detection of resistance correlated with voriconazole treatment failure.[8]

DISCUSSION

Prophylaxis directed at Candida

The European Conference on Infections in Leukaemia (ECIL) 5 guidelines on antifungal prophylaxis recommends fluconazole (400mg q24h) when the mould infections are rare and a mould-directed diagnostic approach is in place (B-I).[10] The latter is the case in all centres that were surveyed but the dose of fluconazole varies among centres and is generally lower than was used in most randomized trials (400mg q24h).[11-15] Some studies suggest that lower doses may suffice.[16] Three centres use oral amphotericin B as primary prophylaxis and in others oral amphotericin B is given on top of fluconazole if surveillance cultures remain positive. In a pooled analysis of oral fluconazole versus amphotericin B no significant advantage of either of the two drugs was observed. Data on the efficacy of prophylactic amphotericin B are scarce.[17] According to the EBMT, fluconazole is the drug of choice for the prophylaxis of invasive candidiasis before engraftment in allo-HSCT recipients, and may be started at the beginning or after the end of the conditioning regimen (A-I).[18]

Mould-active prophylaxis

The advantage of primary mould-active prophylaxis with posaconazole was shown in two randomized trials.[13, 14] The Dutch guideline on antifungal management as well as the ECIL-5 guideline recommends posaconazole for primary prophylaxis (A-I) when the incidence of mould infections is high.[10, 19] 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 local prevalence of mould infections are often lacking.[20] One centre administers aerosolized L-AmB twice weekly for the prevention of IFD in AML patients undergoing intensive chemotherapy. Its efficacy and cost-effectiveness have been demonstrated in a single-centre randomized placebo-controlled trial and an observational study.[21, 22] One centre uses itraconazole as antifungal prophylaxis. A major concern of itraconazole is its poor gastrointestinal tolerance and *CYP3A4* inhibitory properties. Both the ECIL-5 and the IDSA guidelines give moderate recommendations against its use.[10, 23] All centres use a diagnostic protocol that includes a lung CT after three to five days of fever despite antibiotic therapy and proceed to BAL sampling when infiltrates are documented. Indeed, a survival benefit of azole prophylaxis compared with a diagnostic-driven approach has not been convincingly shown and so both continue to be reasonable strategies.

Mould-active prophylaxis in GVHD

Antifungal prophylaxis has been established as standard of care after allo-HSCT with grade II or higher GVHD, but issues concerning drug-drug interactions and factors compromising bioavailability have to be considered. Ullman *et al*. performed a randomized trial in which fluconazole and posaconazole oral solution were compared as fungal prophylaxis in patients with GVHD. Posaconazole prevented IA and resulted in lower numbers of deaths related to IFD although the overall mortality did not differ.[14] All centres administer azole prophylaxis (4 posaconazole, 2 voriconazole, 2 itraconazole) to patients with GVHD of grade II or higher in accordance with ECIL-5 recommendations in which an A-I recommendation is given for posaconazole and a B-I to itraconazole and voriconazole.[10]

Diagnosis of IA

Pulmonary imaging with high-resolution CT (HRCT) was shown to accelerate and improve the diagnosis of IA.[23] The IDSA guideline advocates imaging with chest CT when a patient is suspected to have IA. IDSA guidelines also encourage BAL since signs, symptoms or imaging by itself are often aspecific. All centres use HRCT and BAL as the standard diagnostic procedure. Serum GM monitoring has a moderate sensitivity of ±70% but is insensitive in non-neutropenic patients and the specificity has varied between studies.[23, 24] Only one centre routinely monitors serum GM in patients with prolonged neutropenia. All centres measure BAL-GM and *Aspergillus* DNA PCR is performed in 3 centres on BAL fluid samples). The clinical implementation of PCR-based diagnosis was debated, though not recommended for routine clinical practice in the 2016 IDSA guidelines as few assays have been standardized and well validated.[23]

Susceptibility testing

Azole resistance was rare in The Netherlands before the year 2000 but its prevalence has continued to increase since then.[25] It is currently based on a limited number of resistance-associated mutations (RAMs) in the *cyp51A*-gene (TR_{34}/L98H, TR_{53}, and TR_{46}/Y121F/T289A) and is most likely caused by the environmental use of azole fungicides. [7, 26, 27] The TR_{34}/L98H and TR_{46}/Y121F/T289A accounted for 83% of resistance mutations in 2016.[6] IDSA guidelines do not recommend standard susceptibility testing but these guidelines cannot be applied to The Netherlands.[23, 28] Case series indicate that IA caused by azole-resistant *Aspergillus,* is associated with a very high mortality. [5, 8] The diagnostic tools used for the detection of azole resistance vary from centre to centre. Most perform agar-based screening assays for resistance (VIPcheck™ testing). Phenotypic azole resistance testing according to the EUCAST method is performed by the National mycology reference laboratory only (RefLab). Four centres directly send *Aspergillus* strains to the RefLab and three await the result of the screening assay.

Only very recently, the clinical validity and relevance of PCR-based susceptibility testing on BAL was demonstrated and may explain the limited uptake of resistance detection by PCR at the time of the survey. The AsperGenius® qPCR is a multiplex PCR and can detect the presence of *Aspergillus* DNA and in addition detect the 2 mutations described above. [8, 29] In a recent study the diagnostic performance was evaluated on BAL-samples in 201 patients.[8, 29] The *Aspergillus* BAL qPCR, had a sensitivity of 89% and a specificity of 89% and was able to detect *A. fumigatus* that carried resistance-associated mutations (RAM) in the majority of patients, even in culture-negative BAL. Furthermore, this study showed that response to voriconazole therapy, when given to patients infected with a resistant *A. fumigatus* was poor.[8]

Treatment

The ECIL-6 guideline attributes an A-I recommendation to both voriconazole and isavuconazole for the treatment of IA [30]. Unfortunately, in 2016 surveillance data showed that triazole resistance was present in 101 of 784 (12.9%) patients with a positive *A. fumigatus* culture.[6] These data are based on clinical isolates and it is uncertain what fraction of these patients met EORTC/MSG criteria. However, the clinical relevance of azole resistance in patients with an invasive *Aspergillus* infection was described in a recent multicenter study and small case series have reported a very high mortality in patients infected with a voriconazole resistant *A. fumigatus* that received initial therapy with voriconazole.[5, 8] The management of IA in The Netherlands in the context of a progressively rising incidence of IA caused by azole-resistant *A. fumigatus* strains is challenging because evidence-based data on the most appropriate management of this emerging clinical problem are lacking. At the time of the survey, all centres start voriconazole when the patient is respiratory and clinically stable while awaiting

culture and/or resistance PCR results. In a clinically unstable patient, five centres still start voriconazole, one centre starts posaconazole and another centre starts L-AmB. The feasibility to perform a BAL (and thus cultures) is the decisive factor for one centre. In 2015 an international consensus paper on the management of IA caused by azole-resistant *Aspergillus* isolates advised L-AmB or echinocandin-voriconazole combination as treatment of choice in regions with environmental triazole resistance rates of *Aspergillus* exceeding 10%.[25]

TDM was systematically used in four centres for voriconazole, on indication or not at all in two centres each. Although some studies suggest a relation between voriconazole serum levels and the incidence of adverse events, randomized clinical trials that convincingly show the value of TDM are still lacking.[31]

Off-guideline management (as compared with the Dutch guideline on fungal infections) was observed in some of the centres.[19] One common reason for a delay in policy change after new convincing evidence was published and incorporated in guidelines is the absence of a dedicated haematologist with special interest in infectious diseases and supportive haematological care who critically assesses the local practice on a regular basis. We asked the centres for the reasons of their off-guideline policies and the following answers were given: The continued use of itraconazole instead of posaconazole as anti-mould prophylaxis in 2 centres was driven by the higher costs of other azoles. Both centres recently moved to voriconazole after it became available as a generic drug. One centre preferred voriconazole over posaconazole and this was driven by the unpredictable absorption of the oral solution and the lack of an intravenous formulation of posaconazole when it first came on the market. Another centre used nebulised liposomal amphotericin-B as anti-mould prophylaxis and did this based on locally generated evidence that supports its (cost-)effectivity.[21, 22] Finally, the continued use of oral amphotericin-B solution as anti-yeast prophylaxis (on top of fluconazole) was driven by the fact that it is a harmless intervention (as no systemic toxicity occurs with a non-absorbed drug) and because with this policy, the incidence of candidemia had been very low with this policy for more than 15 years. Therefore, these centres were reluctant to change a safe policy that seems to be very efficacious.

Protocol (figure 1)

Following this survey, a consensus meeting was organised with representatives of all 8 centres and led to the development of a standardized diagnostic and therapeutic protocol on the management of IFD in haematology patients. This protocol was developed in collaboration with the recently established Dutch-Belgian Mycosis Study Group (DB-MSG) and was implemented in all academic haematology centres in 2017 with the goal to gather evidence on the optimal approach towards IFD in the context of azole resistance (The Azole Resistance MANagement Study (AzoRMan) or DB-MSG 002 study,

NCT03121235). The study aims to demonstrate that the use of resistance testing by PCR on BAL fluid from haematology patients with suspected IA will lead to an improved outcome by detecting resistance earlier and changing triazole therapy to L-AmB as soon as resistance is detected. Indeed, the majority of cases of IA remain culture negative and therefore, the use of resistance testing by PCR is considered crucial.[8, 32] The AzoRMan-study is schematically depicted in figure 1 and further information available at www.clinicaltrials.gov. In brief, treatment is based on the documentation of azole susceptibility or resistance and step-down treatment options for patients treated for documented or presumed azole resistance are given.

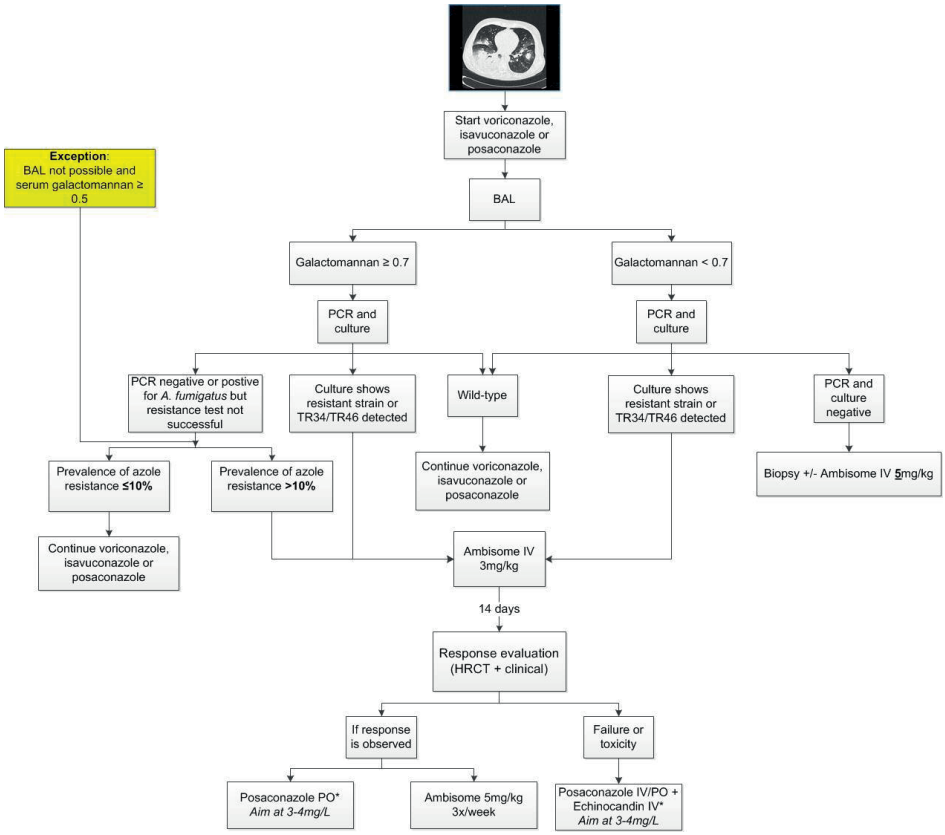

Figure 1: Treatment protocol for Azole-resistance Management-study.
MIC, Minimal Inhibitory concentration; IV, Intravenously. *Posaconazole HD can only be considered as treatment option when the MIC (EUCAST) ≤1 g/dL. HRCT, High Resolution CT scan; PCR, polymerase chain reaction; PO, by mouth; BAL, Broncho-alveolar lavage

Treatment with L-AmB is advised when azole-resistance is documented or when no susceptibility data are available and the local azole-resistance rate is >10%. This is supported by the fact that the *A. fumigatus* strains with the environmental TR_{34}/L98H or the

TR_{46}/Y121F/T289A mutation pattern circulating in the Netherlands remain susceptible to L-AmB.[33] The activity of L-AmB was also confirmed in vivo in immunocompetent and immunosuppressive murine models of IA.[34] This approach may be less appropriate in different settings in which resistance mechanisms other than the environmental TR_{34}/L98H or the TR_{46}/Y121F/T289A mutation patterns are predominant.[35]

If a treatment response is observed during therapy with L-AmB 3mg/kg/day, a switch to L-AmB 5 mg/kg/day three times a week or to oral posaconazole (when the posaconazole MIC is below 2mg/L) is made with a posaconazole target trough serum level of 3 to 4 mg/L. The logical behind the posaconazole strategy is the observation that *Aspergillus* strains carrying RAMs often have a posaconazole minimum inhibitory concentrations (MIC) that is <2mg/L.[36] The efficacy of posaconazole at high serum levels was demonstrated in a pharmacodynamic study in mice with invasive azole-resistant aspergillosis by Mavridou *et al*.[37] This study showed that posaconazole retains activity against an *A. fumigatus* strain that carried the TR_{34}/L98H mutation with a posaconazole MIC of 0.5 mg/L as long as serum drug levels are sufficiently high. No human data on the use of this treatment strategy have been published. However, in a phase 3 pharmacokinetics and safety study for posaconazole tablets the average serum concentration of posaconazole in quartile 4 of the 186 patients that received posaconazole tablets at 300mg per day was 2.3-9.5 mg/L. It was not associated with a specific safety signal and therefore, a serum level between 3 and 4 mg/L is a realistic target.[38] Posaconazole with high serum trough levels is the only oral step-down treatment option for patients with azole-resistant IA. Although clinical evidence remains anecdotal, preclinical animal studies and experience in veterinary medicine provides proof op principle in its efficacy.[37, 39]

CONCLUSION

This survey shows the heterogeneous landscape in the prevention, diagnosis and treatment of IA in The Netherlands. In the context of the rapidly increasing prevalence of azole resistance, the AzorMan study was implemented to evaluate a uniform diagnostic and therapeutic approach.

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Chapter 3

Voriconazole resistance and mortality in invasive aspergillosis: A multicenter retrospective cohort study

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Als we wisten wat we deden, heette het geen onderzoek. (A. Einstein)

ABSTRACT

Background

Triazole resistance is an increasing problem in invasive aspergillosis (IA). Small case series show mortality rates of 50%-100% in patients infected with a triazole-resistant *Aspergillus fumigatus*, but a direct comparison with triazole-susceptible IA is lacking.

Methods

A 5-year retrospective cohort study (2011-2015) was conducted to compare mortality in patients with voriconazole-susceptible and voriconazole-resistant IA. *A. fumigatus* culture-positive patients in three University Medical Centers were investigated to identify patients with proven, probable and putative IA. Clinical characteristics, day-42 and day-90 mortality, triazole resistance profiles and antifungal treatments were investigated.

Results

Of 196 patients with IA, 37 (19%) harbored a voriconazole-resistant infection. Hematological malignancy was the underlying disease in 103 (53%) patients, and 154 (79%) patients were started on voriconazole. Compared with voriconazole-susceptible cases, voriconazole resistance was associated with an increase in overall mortality of 21% on day-42 (49% versus 28%, p=0.017) and 25% on day-90 (62% versus 37%, p=0.0038) corresponding with a hazard ratio of 1·4 (day-42 95%CI 0.8 to 2.5; p=0.272). In non-ICU patients a 19% lower survival rate was observed in voriconazole-resistant cases at day-42 (p=0.045). The mortality in patients receiving appropriate initial voriconazole therapy was 24% compared with 47% in those receiving inappropriate therapy (p=0.016), despite switching to appropriate antifungal therapy after a median of 10 days.

Conclusions

Voriconazole resistance was associated with an excess overall mortality of 21% at day-42 and 25% at day-90 in patients with IA. A delay in the initiation of appropriate antifungal therapy was associated with increased overall mortality.

Triazoles are the mainstay of therapy for invasive aspergillosis (IA) and have led to a substantial improvement in overall survival. However, triazole resistance has become a concern for the management of infections caused by *Aspergillus fumigatus*. Through culture-based surveillance studies the number of countries that report azole resistance continues to increase, although resistance frequencies vary considerably between different geographic regions [1]. Resistance rates as high as 29% have been observed in specific patient populations, such as critically-ill patients [2]. Variations in resistance

frequencies may reflect true geographical differences or might be due to other variables, including study design, patient populations and laboratory practices [3,4].

Triazole resistance may develop through therapy of individual patients with *Aspergillus* disease primarily occurring in patients with chronic pulmonary aspergillosis [5]. More important, triazole resistance may develop in the environment following exposure to azole fungicides [6]. Patients inhale *A. fumigatus* spores resistant to medical triazoles, which may evolve into triazole-resistant IA. The environmental route is characterized by an apparent lack of patient risk factors as the majority of patients who present with triazole-resistant IA have not been previously treated with medical triazoles [7]. The optimal management of patients suspected of IA in regions with environmental resistance remains unclear, and an expert panel recommend considering moving away from triazole monotherapy when regional resistance frequencies exceed 10% [8]. This 10% threshold has been the subject of debate given the toxicity, costs, lack of oral formulations and of comparative clinical trials of non-triazole antifungals like liposomal amphotericin B (L-AmB) and echinocandins or antifungal combination therapies. Animal experiments consistently show that the efficacy of triazoles in infection with *A. fumigatus* with elevated triazole MICs is reduced compared with wild-type infection [9,10]. This has been shown for itraconazole, voriconazole, posaconazole and isavuconazole. Furthermore, several small case series reported mortality rates of 50%-100% in patients with triazole-resistant IA [11]. These rates are higher than those reported in recent clinical trials, where mortality rates in triazole-treated aspergillosis patients were below 30%, but selection bias may partially explain the very high mortality and therefore, the exact impact of triazole resistance remains to be defined as direct comparisons between triazole-susceptible and triazole-resistant infection are lacking [7,12]. To investigate the characteristics and outcome of voriconazole-susceptible IA and voriconazole-resistant IA, we conducted a retrospective, multicentre study in a large cohort of *A. fumigatus* culture-positive patients.

METHODS

Study design

A retrospective cohort study was performed in three tertiary care University Medical Centers in the Netherlands: Radboud University Medical Center in Nijmegen, Leiden University Medical Center in Leiden and Erasmus University Medical Center in Rotterdam.

General management of IA

Diagnostic work-up in patients suspected of invasive pulmonary mould disease, typically included chest CT and, if possible, bronchoscopy and bronchoalveolar lavage (BAL). In patients with acute myeloid leukemia, myelodysplastic syndrome, and hematopoietic stem cell transplant recipients a diagnostic driven strategy was used including monitoring of serum galactomannan (GM) during neutropenia or in febrile patients. Chest CT was performed in patients with positive serum GM, in those with persistent fever despite three to five days of broad-spectrum antibacterial therapy, and in patients with progressive respiratory failure. If CT confirmed the presence of pulmonary infiltrates, a BAL was performed for fungal culture and GM measurement. Voriconazole was the first choice treatment option for patients with IA. During the study period no hospital treatment guidelines were available for documented voriconazole-resistant IA, but when resistance was documented or suspected in critically-ill patients, treatment was changed to either triazole and echinocandin combination therapy or L-AmB.

Data collection

The microbiology database was searched for positive *A. fumigatus* cultures of patients admitted between January 2011 and December 2015. In order to select patients with IA the clinical records of culture-positive patients needed to meet three conditions: (i) antifungal therapy was started within one month before or after a positive culture, (ii) the patient had received at least two days of antifungal therapy, and (iii) the patient could be classified as probable or proven IA according to EORTC/MSG definitions or putative or proven according to AspICU criteria for the subgroup of patients admitted to the ICU [13,14].

Patient characteristics included age, gender, underlying diseases, ward/ICU admission, and antifungal prophylaxis or therapy. ICU admission was defined as initiation of antifungal therapy in the ICU and stay in the ICU for at least two consecutive days. In addition, patients who were admitted to the ICU during their hospitalization were analyzed separately in a cox regression model. Furthermore, the appropriateness of initial antifungal therapy was assessed for patients treated with voriconazole. Antifungal therapy was considered appropriate if voriconazole was started in patients with

voriconazole-susceptible disease and inappropriate in those with voriconazole-resistant IA. Switch to appropriate antifungal therapy and time to switch were determined.

The study was reviewed by the Institutional Review Boards, which confirmed that the study did not fall under the Dutch law on research on human subjects. Data were processed after encoding and in accordance with the Dutch Personal Data protection act.

Mycology

Fungal cultures were routinely performed if a patient underwent bronchoscopy with BAL and if ordered for other respiratory specimens. *A. fumigatus* was identified by macroscopic and microscopic morphology and growth at 48°C. *A. fumigatus* isolates were routinely screened for the presence of triazole resistance using an agar-dilution method (VIPcheck™, Nijmegen, the Netherlands) [15]. The method relies on agar-wells supplemented with itraconazole, voriconazole and posaconazole and a growth control. Fungal growth on any triazole-containing well was considered indicative of resistance and these isolates were sent to Radboud University Medical Center for MIC-testing according to the EUCAST reference method [16]. Infection was considered to be voriconazole-resistant if one or more cultured *A. fumigatus* isolates exhibited a voriconazole MIC above the clinical breakpoint of 2 mg/l. If a patient had more isolates cultured within one month of initiation of antifungal therapy, the most resistant isolate was used to classify the patient. In addition, the presence of a resistance mutation in cyp51A was determined by *Cyp51A*-gene sequencing, which is specific for *A. fumigatus* sensu strictu, excluding sibling species from the *A. fumigatus* species complex [17,18].

Data analysis

The primary endpoints were day-42 and day-90 mortality in voriconazole-resistant IA compared with voriconazole-susceptible IA cases. Day zero was set at day of initiation of antifungal therapy. Other factors with possible impact on survival were also investigated including choice of first-line antifungal therapy, ICU-admission, Acute Physiology And Chronic Health Evaluation II (Apache II) score, and appropriateness of initial antifungal therapy.

Statistical analysis

Statistical analysis on the relation of voriconazole resistance and mortality was performed in SAS® 9.4 and SPSS® 24 with survival analysis (Kaplan-Meier) and the log-rank method. Confidence intervals were calculated with the Kaplan-Meier method. Possible confounders, i.e. ICU admission, underlying hematological disease and center, were analyzed for each comparison with Cox regression survival analysis, Kaplan-Meier survival (Log rank) and Fisher exact. Other differences were compared with Fisher exact.

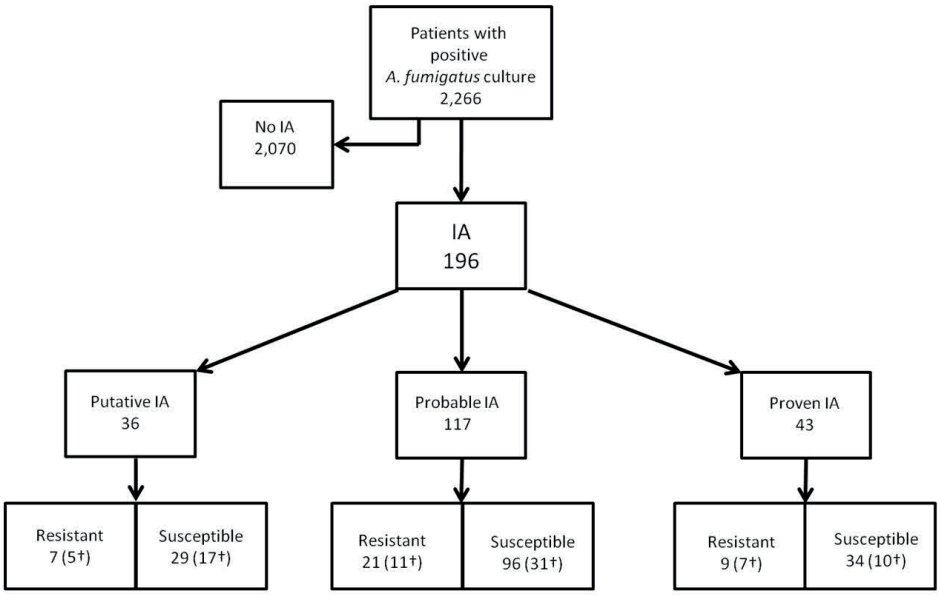


Figure 1: Inclusion of patients with positive *A. fumigatus* cultures from lower respiratory tract or sterile specimens between January 2011 and December 2015. 2,266 patients had one or more positive cultures, and 196 patients could be classified according to the definitions of AspICU and EORTC/MSG. IA, invasive aspergillosis; †, mortality.

RESULTS

Demographics

In the five-year period 2,266 patients with a positive *A. fumigatus* culture in the three centers were eligible for the study. Overall, 196 (8.6%) patients met our case definition, i.e. received antifungal therapy within 30 days of a positive culture, received at least two days of antifungal therapy and could be classified according to the EORTC/MSG or AspICU criteria (Figure 1). A proven infection was documented in 43 (22%) patients, a putative diagnosis in 36 (18%) patients and a probable diagnosis in 117 (60%) patients (Table 1). Hematological malignancy was the most frequent underlying disease diagnosed in 103 of 196 (53%) patients. Eighty-five (43%) patients were admitted to the ICU during hospital admission, while 59 (30%) patients first received antifungal therapy in the ICU. Voriconazole was the initial therapy in 154 (79%) patients. Further details regarding the demography for individual centers and the total patient population are shown in Table 1.

Voriconazole susceptibility

Voriconazole resistance was observed in 37 of 196 (19%) patients, but the resistance frequency varied between 10% and 31% in individual centers (Table 1). Voriconazole-

Parameter[a]		Center 1 (60 cases)	Center 2 (59 cases)	Center 3 (77 cases)	Total (196 cases)
Patient	Male / Female	34 / 26	36 / 23	45 / 32	115 / 81
	Median age	64 (3 – 79)	61 (4 – 80)	61 (2 – 83)	62 (2-83)
	Hematological malignancy	23 (38%)	34 (58%)	46 (60%)	103 (53%)
	Autoimmune disease	12 (20%)	6 (10%)	6 (8%)	24 (12%)
	Solid organ transplant	8 (13%)	5 (8%)	11 (14%)	24 (12%)
	Structural lung disease	5 (8%)	6 (3%)	3 (4%)	14 (7%)
	Solid tumor	3 (5%)	-	3 (4%)	6 (3%)
	Congenital immune disorder	4 (7%)	-	-	4 (2%)
	Other	4 (7%)	8 (4%)	2 (3%)	14 (7%)
	None	1 (2%)	-	6 (8%)	7 (4%)
	ICU-admission	20 (33%)	23 (39%)	16 (21%)	59 (30%)
	APACHE II-score	21	25	21	22
IA	Putative	15 (25%)	14 (24%)	7 (9%)	36 (18%)
	Probable	24 (40%)	39 (66%)	54 (70%)	117 (60%)
	Proven	21 (35%)	6 (10%)	16 (21%)	43 (22%)
	Voriconazole-susceptible	54 (90%)	41 (69%)	64 (83%)	159 (81%)
	Voriconazole-resistant	6 (10%)	18 (31%)	13 (17%)	37 (19%)
ICU	Voriconazole-susceptible	18 (90%)	17 (74%)	10 (63%)	45 (76%)
	Voriconazole-resistant	2 (10%)	6 (26%)	6 (37%)	14 (24%)
non-ICU	Voriconazole-susceptible	36 (90%)	24 (67%)	54 (89%)	114 (83%)
	Voriconazole-resistant	4 (10%)	12 (33%)	7 (11%)	23 (17%)
Management	Triazole prophylaxis	-	16 (27%)	4 (5%)	20 (10%)
	Voriconazole	42 (70%)	45 (76%)	67 (87%)	154 (79%)
	Itraconazole	1 (2%)	-	-	1 (0.5%)
	Posaconazole	1 (2%)	-	2 (3%)	3 (2%)
	L-AmB	13 (22%)	13 (22%)	1 (1%)	27 (14%)
	Echinocandin	-	-	1 (1%)	1 (0.5%)
	VCZ+L-AmB	-	-	4 (5%)	4 (2%)
	VCZ+Ecand	3 (5%)	1 (2%)	1 (1%)	5 (3%)
	VCZ+intrathecal caspofungin/L-AmB	-	-	1 (1%)	1 (0.5%)
	Inappropriate therapy / appropriate therapy	3 / 57	15 / 44	12 / 65	30 /196
Outcome	42-day mortality	13 (22%)	26 (65%)	23 (30%)	62 (32%)
	90-day mortality	23 (38%)	29 (85%)	29 (38%)	81 (42%)
	42-day mortality ICU	11 (55%)	18 (78%)	7 (44%)	36 (61%)
	90-day mortality ICU	13 (65%)	20 (87%)	7 (44%)	40 (68%)
	42-day mortality non-ICU	2 (3%)	8 (14%)	15 (19%)	25 (18%)
	90-day mortality non-ICU	10 (17%)	9 (15%)	22 (29%)	41 (30%)

Table 1: Demographics, invasive aspergillosis classification, voriconazole susceptibility, management and outcome of 196 patients with culture-positive invasive aspergillosis.
[a]L-AmB, liposomal-amphotericin B; VCZ, voriconazole.

resistant IA was diagnosed in 14 of 59 (24%) ICU patients and in 23 of 137 (17%) non-ICU patients. Voriconazole resistance corresponded with resistance to isavuconazole for all 14 patients where isavuconazole susceptibility was determined (Table 2). In 30 of 37 (81%) patients the *A. fumigatus* isolate showed a pan-triazole resistant phenotype, while in seven patients the susceptibility to itraconazole, voriconazole and posaconazole varied. Analysis of triazole-resistant *A. fumigatus* isolates showed resistance mutations that are associated with the environmental route of resistance selection in 32 of 37 (87%) patients; TR_{34}/L98H in 18 patients and TR_{46}/Y121F/T289A in 13 patients (Table 2). In five voriconazole-resistant isolates no mutations were found in the *Cyp51A*-gene, suggesting that other uncharacterized resistance mutations might be present. In seven patients (19%) a mixed infection was diagnosed; this consisted of an infection with a triazole-resistant and susceptible *A. fumigatus* in six patients (S/R), while in one patient isolates with two different resistance mutations were recovered (R/R; Table 2). All cultured *A. fumigatus* isolates were susceptible to amphotericin B.

Mortality

The overall mortality in the 195 patients with IA was 62 (32%) at day-42 and 81 (42%) at day-90 (Table 1). One patient was discharged to a hospice on day-25 but the exact day of death was not known, therefore his survival was censored at 25 days. Comparing the patients infected with voriconazole-susceptible and voriconazole-resistant *A. fumigatus*, a 21% higher overall mortality was observed in patients infected with a resistant isolate; 44 of 158 (28%; 95%CI 21% to 35%) patients with voriconazole-susceptible infection had died at day-42 versus 18 of 37 (49%; 95%CI 34% to 66%; log-rank test, p=0.017) of those with voriconazole-resistant IA. At day-90 the absolute difference in mortality had increased to 25% (58 of 158; 37%; 95%CI 30% to 45% and 23 of 37; 62%; 95%CI 47% to 77%, respectively; log-rank test, p=0.0038; Figure 2A). As expected, the cumulative survival rates were much lower for 59 patients who first received antifungal therapy in the ICU; mortality was 26 of 45 (58%) for patients with voriconazole-susceptible IA and 10 of 14 (71%) for those with voriconazole-resistant IA at day-42 (log-rank test, p=0.37; Figure 2B). For 136 patients who first received antifungal therapy on the ward (non-ICU group) a 19% lower survival rate was observed for patients with voriconazole-resistant IA, compared with voriconazole-susceptible infection, with a mortality rate of 8 of 23 (35%) and 19 of 113 (16%) at day-42, respectively (log-rank test, p=0.045; Figure 2C). The mortality for 18 patients infected with TR_{34}/L98H was not different from 13 patients with TR_{46}/Y121F/T289A infection (supplementary Figure S1).

At the discretion of the treating physician, 27 of 196 (14%) patients received initial antifungal therapy with L-AmB. Eight of these 27 patients were infected with voriconazole-resistant *A. fumigatus*. The survival at day-42 of L-AmB treated patients was 55% compared with 71% for voriconazole-treated patients (log-rank test, p=0.04; Figure 3).

Sex, age	Center	Underlying disease[¤]	ICU	IA classification	Sample with resistant culture	Cyp51A-resistance mutation[¥]	MIC (mg/l)[¤]				Initial antifungal therapy[§]	Outcome at day 90 (day of death)
M, 49	1	Diabetes, necrotizing pancreatitis	Yes	Proven	Lung biopsy	TR_{34}/L98H	>16	16	1	8	L-AmB	Alive
M, 71	1	Kidney transplant	No	Proven	Bronchial aspirate	TR_{46}/Y121F/T289A[*S/R]	0.5	>16	0.25	>16	VCZ	Died (+50)
M, 55	1	AML, alloHSCT	No	Probable	Sputum	WT	1	4	0.25	-	L-AmB	Died (+52)
M, 70	1	COPD, lung fibrosis	Yes	Putative	BAL	WT	>16	>16	1	-	VCZ	Died (+14)
F, 59	1	T cell lymphoma	No	Proven	Sinus biopsy	TR_{34}/L98H	>16	4	0.5	-	L-AmB	Died (+15)
F, 65	1	Kidney transplant	No	Proven	Brain biopsy	TR_{34}/L98H[*S/R]	>16	8	0.5	8	VCZ	Died (+90)
F, 69	2	MDS	Yes	Probable	BAL	TR_{46}/Y121F/T289A	>16	>16	1	-	L-AmB	Alive
M, 51	2	B cell lymphoma	No	Probable	Sputum	TR_{34}/L98H	>16	8	0.5	-	VCZ	Alive
F, 64	2	B cell lymphoma	Yes	Probable	BAL	TR_{46}/Y121F/T289A	>16	>16	1	-	L-AmB	Died (+25)
M, 59	2	AML	Yes	Putative	BAL	TR_{34}/L98H	16	8	1	-	VCZ	Died (+27)
F, 39	2	Liver transplant	Yes	Putative	BAL	TR_{34}/L98H	>16	16	1	-	VCZ	Died (+62)
F, 44	2	Auto immune hepatitis	Yes	Putative	BAL	TR_{46}/Y121F/T289A	>16	>16	1	-	VCZ	Died (+7)
M, 61	2	B cell lymphoma	No	Probable	Sputum	TR_{46}/Y121F/T289A[*R/R]	>16	>16	1	-	L-AmB	Died (+11)
					BAL	TR_{34}/L98H	>16	8	1	-		
M, 15	2	Severe aplastic anaemia	Yes	Probable	BAL	TR_{34}/L98H	>16	8	1	-	L-AmB	Died (+4)
F, 53	2	Lung carcinoma	Yes	Probable	Drain fluid	TR_{34}/L98H	>16	4	0.5	-	VCZ	Died (+7)
M, 67	2	COPD	Yes	Proven	Lung autopsy	TR_{34}/L98H	>16	8	1	-	VCZ	Died (+8)
F, 19	2	B cell lymphoma	No	Probable	Sputum	TR_{34}/L98H	>16	8	1	16	VCZ	Died (+18)
M, 54	2	B cell lymphoma	No	Probable	BAL	TR_{34}/L98H[*S/R]	>16	8	1	8	VCZ	Alive
M, 45	2	B cell lymphoma	No	Probable	Bronchial aspirate	TR_{46}/Y121F/T289A[*S/R]	1	>16	0.25	>16	VCZ	Alive
F, 67	2	AML	No	Probable	Bronchial aspirate	TR_{46}/Y121F/T289A	>16	>16	0.25	>16	VCZ	Alive
M, 62	2	B cell lymphoma, alloHSCT	No	Proven	Tissue biopsy	TR_{46}/Y121F/T289A	0.5	>16	0.5	>16	VCZ	Alive
M, 71	2	Influenza	Yes	Putative	Sputum	TR_{34}/L98H[*S/R]	>16	>16	2	>16	VCZ	Alive
M, 70	2	AML	Yes	Probable	Bronchial aspirate	TR_{34}/L98H	>16	8	1	8	VCZ	Alive

Sex, age	Center	Underlying disease[¤]	ICU	IA classification	Sample with resistant culture	Cyp51A-resistance mutation[¥]	MIC (mg/l)[¤]				Initial antifungal therapy[§]	Outcome at day 90 (day of death)
F, 58	2	Follicular lymphoma	Yes	Putative	BAL	WT[*S/R]	>16	>16	1	>16	VCZ	Alive
M, 22	3	alloHSCT	Yes	Probable	Sputum	TR_{46}/Y121F/T289A	>16	>16	1	-	L-AmB	Died (+9)
M, 46	3	Kidney Tx, Marginal zone lymphoma	No	Probable	BAL	TR_{34}/L98H	>16	4	0.5	8	VCZ	Alive
M, 53	3	Haematological malignancy	No	Probable	Sputum	TR_{46}/Y121F/T289A	>16	>16	1	-	VCZ	Died (+66)
F, 64	3	Haematological malignancy	No	Probable	Sputum	TR_{34}/L98H	>16	16	2	-	VCZ	Alive
M, 64	3	Haematological malignancy	No	Probable	BAL	TR_{46}/Y121F/T289A	>16	4	1	-	VCZ	Alive
M, 29	3	Hodgkin lymphoma, alloHSCT	Yes	Probable	BAL	TR_{34}/L98H	>16	8	2	-	VCZ	Died (+44)
F, 63	3	Lung transplant	Yes	Probable	Sputum	TR_{34}/L98H	>16	16	2	16	VCZ	Died (+40)
F, 64	3	Neurosarcoidosis	Yes	Probable	Sputum	WT	0.25	4	0.25	-	VCZ	Died (+4)
F, 40	3	alloHSCT	Yes	Proven	BAL	TR_{34}/L98H	>16	4	1	-	VCZ	Died (+32)
M, 56	3	Autoimmune disease	Yes	Proven	Sputum	TR_{46}/Y121F/T289A	2	>16	1	-	VCZ	Died (+32)
F, 60	3	SOT	Yes	Proven	Sputum	TR_{46}/Y121F/T289A	16	>16	0.5	-	VCZ	Alive
F, 62	3	Hematological malignancy, influenza	Yes	Proven	BAL	TR_{46}/Y121F/T289A	2	>16	1	-	VCZ	Died (+10)
M, 38	3	Poly trauma	Yes	Putative	BAL	WT	>16	4	0.5	8	VCZ	Died (+24)

Table 2: Underlying condition, invasive aspergillosis classification, *A. fumigatus* genotype and phenotype, and outcome in 37 patients with voriconazole-resistant IA.

[¤]AML, acute myeloid leukaemia; AlloHSCT, allogeneic haematopoietic stem cell transplant; COPD, chronic obstructive pulmonary disease; MDS, myelodysplastic syndrome; CLL, chronic lymphoblastic laeukemia; SOT, solid organ transplant.

[¥] WT, wild type.

*Mixed infection; [*S/R] voriconazole-susceptible and voriconazole-resistant; [*R/R] two different voriconazole-resistant genotypes.

[¤]ITZ, itraconazole, VCZ, voriconazole; POS, posaconazole; ISA, isavuconazole.

[§]VCZ, voriconazole, L-AmB, liposomal amphotericin B.

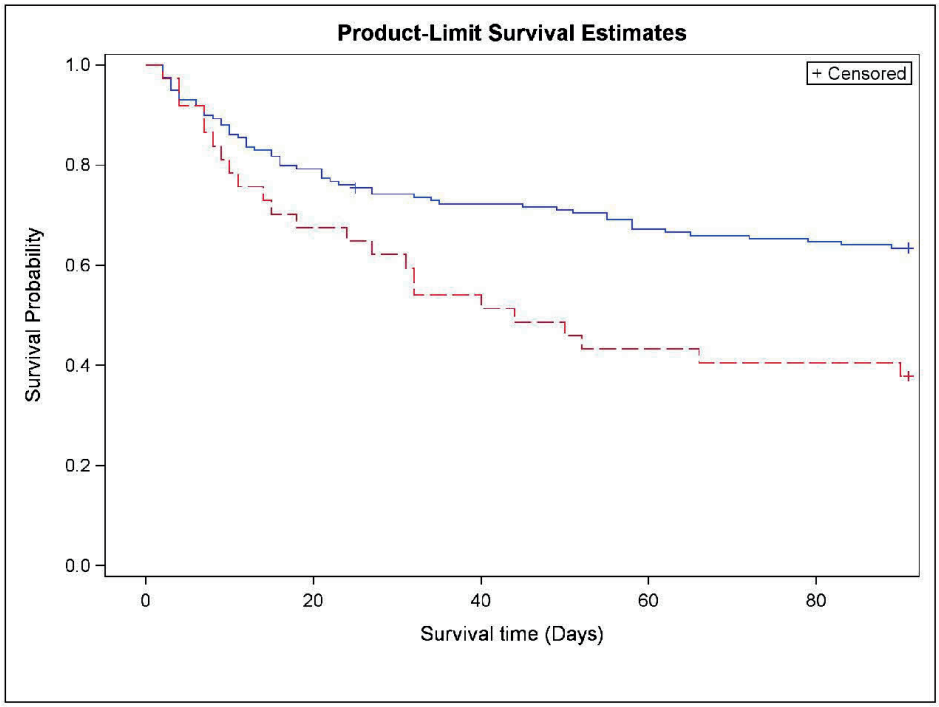


Figure 2A

Group / Day	0	42		90	
VCZ-S	159	114	(72%; CI 65% - 79%)	100	(63%; CI 55% - 70%)
VCZ-R	37	19	(51%; CI 34% - 66%)	14	(38%; CI 23% - 53%)
P-value log rank test			0.017		0.0038

Figure 2: Cumulative survival of patients with voriconazole-susceptible and voriconazole-resistant IA. A. Cumulative survival of all patients with IA. B. Cumulative survival of patients that started antifungal therapy at the ICU. C. Cumulative survival in non-ICU patients with IA. Blue lines represent patients with IA due to voriconazole-susceptible *A. fumigatus* (VCZ-S); Red lines represent patients with IA due to voriconazole-resistant *A. fumigatus* (VCZ-R). One patient was discharged to a hospice after 25 days and his survival was therefore censored at day-25.

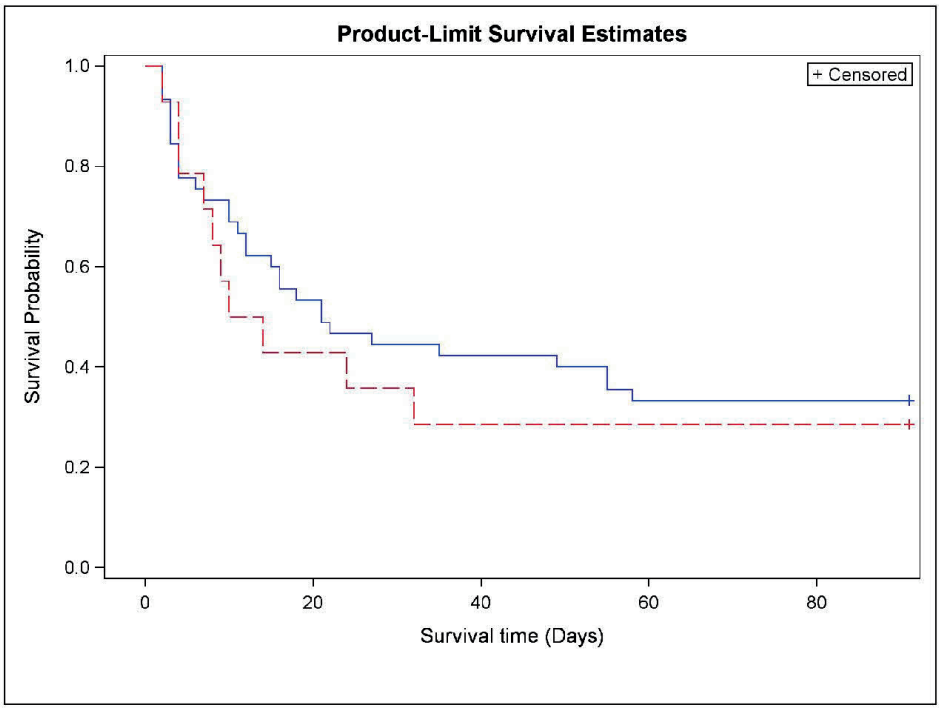


Figure 2B

Group / Day	0	42		90	
VCZ-S	45	19	(42%; CI 28% - 56%)	15	(33%; CI 20% - 47%)
VCZ-R	14	4	(29%; CI 9% - 52%)	4	(29%; CI 9% - 52%)
P-value log rank test			0.37		0.57

Figure 2: Cumulative survival of patients with voriconazole-susceptible and voriconazole-resistant IA. A. Cumulative survival of all patients with IA. B. Cumulative survival of patients that started antifungal therapy at the ICU. C. Cumulative survival in non-ICU patients with IA. Blue lines represent patients with IA due to voriconazole-susceptible *A. fumigatus* (VCZ-S); Red lines represent patients with IA due to voriconazole-resistant *A. fumigatus* (VCZ-R). One patient was discharged to a hospice after 25 days and his survival was therefore censored at day-25.

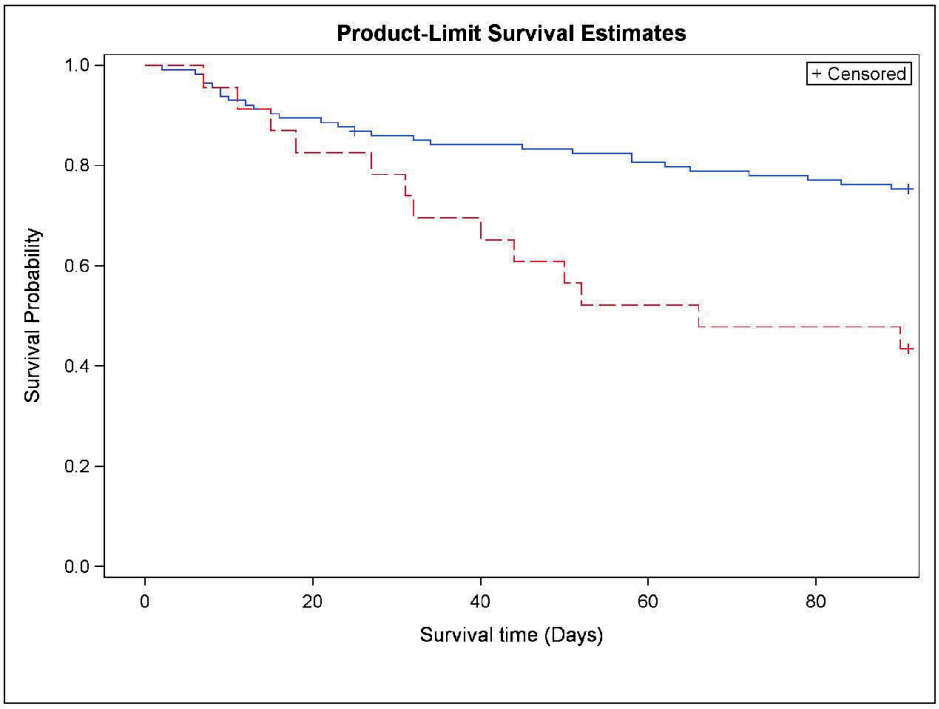
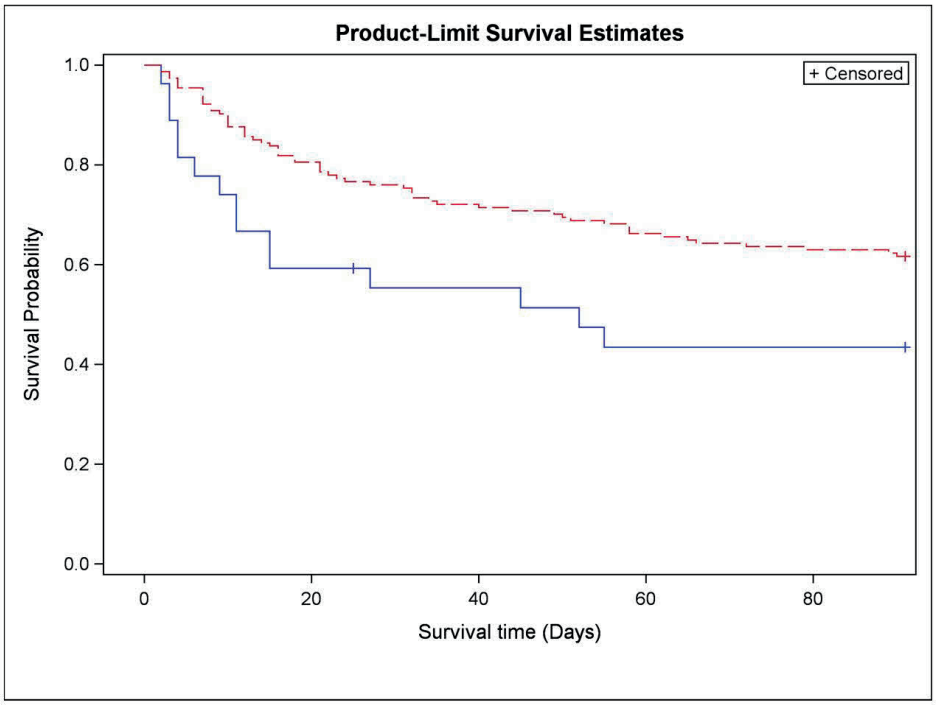


Figure 2C

Group / Day	0	42		90	
VCZ-S	114	95	(84%; CI 76% - 90%)	85	(75%; CI 66% - 82%)
VCZ-R	23	15	(65%; CI 42% - 81%)	10	(43%; CI 23% - 62%)
P-value log rank test			0.045		0.002

Figure 2: Cumulative survival of patients with voriconazole-susceptible and voriconazole-resistant IA. A. Cumulative survival of all patients with IA. B. Cumulative survival of patients that started antifungal therapy at the ICU. C. Cumulative survival in non-ICU patients with IA. Blue lines represent patients with IA due to voriconazole-susceptible *A. fumigatus* (VCZ-S); Red lines represent patients with IA due to voriconazole-resistant *A. fumigatus* (VCZ-R). One patient was discharged to a hospice after 25 days and his survival was therefore censored at day-25.

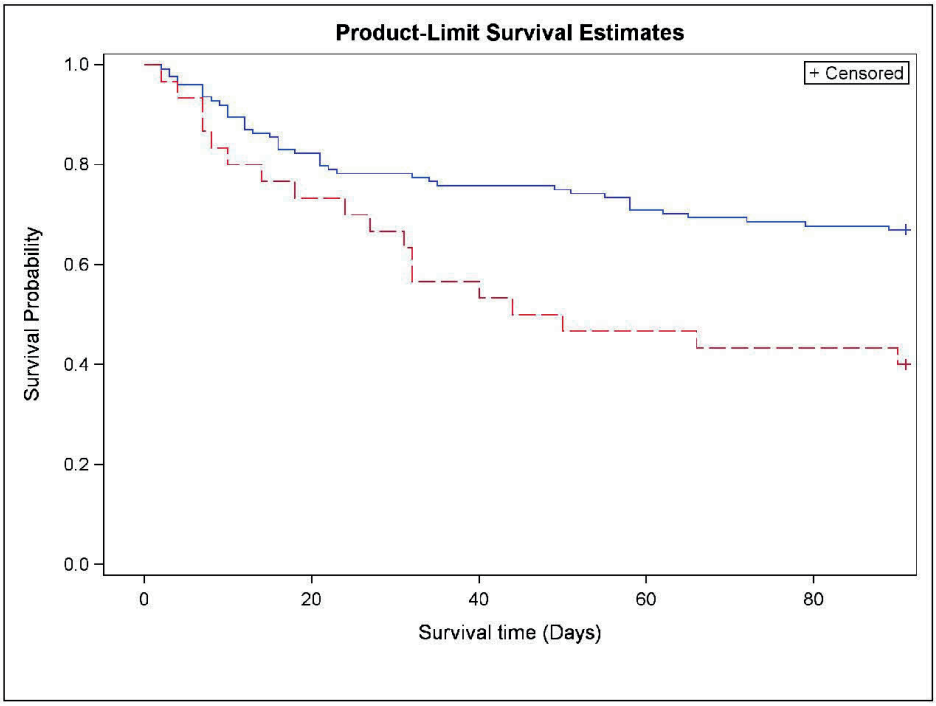


Group / Day	0	42		90	
VCZ	154	110	(71%; CI 64% - 78%)	95	(62%; CI 54% - 69%)
L-AmB	27	14	(55%; CI 35% - 72%)	11	(43%; CI 24% - 61%)
P-value log rank test			0.04		0.025

Figure 3: Cumulative survival of L-AmB-treated IA patients compared with voriconazole-treated patients. Red line represents patients with IA who were treated with voriconazole (VCZ); Blue line represents patients with IA who were treated with liposomal amphotericin B (L-AmB). One patient was discharged to a hospice after 25 days and his survival was therefore censored at day-25.

However, the proportion of ICU-patients in the L-AmB-treated group was significantly higher compared with voriconazole-treated patients (13 of 27 (48%) versus 43 of 154 (28%); Fisher exact test, p=0.04), indicating that confounding by indication at least partly explained this difference.

The mortality of patients receiving appropriate and inappropriate therapy was compared for 154 patients with initial voriconazole therapy. Thirty patients (81%) with voriconazole-resistant IA initially received voriconazole therapy and were classified to have received inappropriate antifungal therapy (Table 2). Therapy was switched to appropriate therapy in 18 patients after a median of 10 days (range 1 to 39 days). Inappropriate voriconazole therapy corresponded with reduced survival at day-42 compared with appropriate therapy (76% and 53%, respectively; log-rank test, p=0.016; Figure 4). Six patients presented with mixed infection (Table 2).



Group / Day	0	42		90	
Appropriate	124	94	(76%; CI 67% - 82%)	83	(67%; CI 58% - 74%)
Inappropriate	30	16	(53%; CI 34% - 69%)	12	(40%; CI 23% - 57%)
P-value log rank test			0.016		0.0049

Figure 4: Cumulative survival of patients that initially received voriconazole therapy; patients receiving appropriate initial voriconazole therapy were compared with those receiving inappropriate therapy. Blue line represents patients with IA who received appropriate initial voriconazole therapy; red line represents patients with IA who received inappropriate initial voriconazole therapy.

Cox regression analysis

ICU admission, underlying hematological disease, and center were analyzed as possible confounders for mortality. ICU admission contributed significantly to mortality, whereas the presence of hematological disease had no effect (see Supplementary Table 1). Comparison of the centers indicated that the resistance frequency was significantly higher in centre 2 in comparison with centers 1 and 3 (p=0.009). The hazard ratio at day-42 for patients who started voriconazole therapy on the ICU was 7.7 (95%CI 3.9 to 15.3; p<0·001), while a hazard ratio of 1.4 was found for voriconazole resistance (95%CI 0.8 to 2.4; p=0·272; Table S1). In patients where voriconazole therapy was initiated on the ward, voriconazole resistance frequency was higher in patients who required ICU-admission compared to those who completed treatment on the ward (8 of 16 (50%) compared with 15 of 111 (14%) patients, respectively; p=0.044).

DISCUSSION

Our retrospective cohort study showed a higher mortality in patients with voriconazole-resistant IA compared with voriconazole-susceptible IA. In a setting of primary therapy with voriconazole, the absolute difference in day-42 and day-90 mortality ranged between 21% and 33%, respectively for the overall patient group and for non-ICU patients. These observations are in line with results from in-vivo models of resistant infection and case series [7,9,10,12,19]. However, these case series included a small number of IA patients and were therefore prone to selection or publication bias. In the subset of patients admitted to the ICU, no significant difference in survival between voriconazole-resistant and voriconazole-susceptible IA was found. However, the smaller sample size of this subgroup as well as the high mortality of 67% in voriconazole-susceptible IA patients in the ICU makes this analysis severely underpowered.

L-AmB is considered alternative treatment for IA but a randomized comparison with voriconazole has never been performed and therefore, its efficacy relative to voriconazole remains unclear [24]. In our study the survival of L-AmB treated patients was not better than voriconazole-treated patients with IA. However, patients that received L-AmB were more often admitted to the ICU compared with patients on voriconazole and therefore had an a priori higher probability of dying. Although the very small number of patients in this subanalysis makes any definite conclusions premature, this may indicate that in critically-ill patients and those with advanced IA the clinical deterioration could not be reversed by polyene-based therapy. Indeed, pre-clinical studies showed that L-AmB, even at a dose of 10 mg/kg, was ineffective when treatment was delayed until 48 hours post-infection [25], underscoring the need for early intervention. Treatment delay was also found to be associated with poorer outcome of IA in clinical studies [26], which is supported by our observation of lower survival when the initial antifungal therapy was inappropriate.

Voriconazole resistance was dominated by mutations associated with environmental resistance selection, accounting for 87% of resistance mutations [7,11,12]. The majority of isolates were pan-azole resistant and there was 100% cross-resistance between voriconazole and isavuconazole. There are no known risk factors that can help to identify patients at high risk for triazole-resistant IA, and in our study all cases of inappropriate antifungal therapy were due to voriconazole therapy in voriconazole-resistant IA.

Our study has several limitations, including its retrospective design. Many factors may impact on the outcome of IA and some of these could act as confounder as they may not be well balanced between voriconazole-susceptible and voriconazole-resistant patient groups. We identified possible confounders in our cohort. As expected ICU-admission was associated with significant higher mortality. However, when ICU-patients were excluded, mortality in voriconazole-resistant IA remained significantly higher compared

with voriconazole-susceptible IA. Furthermore, patients with voriconazole-resistant IA were more likely to require ICU-admission, suggesting that initial therapy was not successful. Cox regression analysis indicated that the hazard of death due voriconazole-resistance was 1.4 times higher than in voriconazole-susceptible infection.

Our study relied on aspergillus culture as this enabled reliable resistance screening and in-vitro susceptibility testing. Agar-based resistance screening through VIPcheck™ was found to be highly sensitive and specific to identify resistant *A. fumigatus* colonies in cultures, and unlike PCR-based resistance detection, allows detection of a broad range of resistance mutations, including uncharacterized mechanisms [15]. However, sensitivity of culture is low and thus our cohort represents a small subset of IA cases and may not be directly translatable to culture negative cases of IA. However, a recent study that used PCR cyp51A resistance testing directly on BAL of hematology patients with IA showed a 31% difference in overall mortality, similar to what we observed [19].

As 79% of patients received initial therapy with voriconazole, our study represents an escalation strategy, i.e. initial voriconazole and escalation when resistance is documented. An escalation strategy is recommended by the IDSA, where MIC-testing is advocated in patients suspected of resistance or failing to primary antifungal therapy [24]. In our study a higher mortality was observed if patients with voriconazole-resistant IA started on voriconazole despite intensive resistance screening. Treatment was switched after a median of 10 days, which did not prevent poor clinical outcome. A management strategy based on less intensive resistance testing, such as recommended by the IDSA, might result in excess mortality in those patients with voriconazole-resistant IA. Direct detection of resistance mutations by molecular techniques in BAL-fluid may reduce the time to resistance detection, and PCR-based strategic studies are currently ongoing.

As appropriate initial antifungal therapy was found to be critical, upfront combination antifungal therapy may be required to increase the probability of survival of patients at risk for IA in geographic regions with high resistance rates. Combination therapy includes voriconazole or isavuconazole combined with an echinocandin or L-AmB, but clinical evidence supporting these treatment options is lacking. However, the 10% threshold recommended by an expert panel was met in our centers, and the Dutch treatment guideline has now been revised recommending routine triazole resistance testing and combination therapy for patients suspected of IA, at least until the presence of resistance has been ruled out [27]. In most countries resistance rates are lower than reported in the Netherlands, which does not justify a de-escalation strategy [1,28].

Our findings underscore the need for rapid resistance tests and antifungal drugs based on new targets. As azole fungicide use appears to be an important driver for resistance in *A. fumigatus* and new resistance mutations continue to emerge in the environment [29], strategies need to be developed aimed at overcoming resistance selection in the environment. However, antimicrobial resistance (AMR) action plans and 'One-Health'

research are generally restricted to bacterial resistance [30]. Governments, medical research councils and public health organizations are called to action to prioritize fungal research and help to overcome the problem of triazole resistance.

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Potential conflicts of Interest

P.E.V. has received financial support from Gilead Sciences, MSD, Pfizer, F2G and non-financial support from OLM and IMMY, outside submitted work. B.J.A.R. received financial support from Gilead Sciences, outside submitted work. A.F.A.D.S. has received financial support from Abvie, Roche, Gilead Sciences and Pfizer, outside submitted work.

P.P.L, R.G.B., S.S., W.J.F.M.v.d.V., E.J.K., J.v.P., B.v.d.H., H.A.v.d.L., W.J.G.M., A.F.d.H., H.L.v.d.H., M.T.v.d.B., No conflict.

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Chapter 4

High-dose posaconazole for azole-resistant aspergillosis and other difficult-to-treat mould infections

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Reference: Mycoses. 2020;63:122–130

There is nothing more deceptive than an obvious fact. (Arthur C Doyle)

ABSTRACT

Background

Oral follow-up therapy is problematic in moulds with reduced azole-susceptibility, such as azole-resistant *A. fumigatus* infection. Currently only intravenous liposomal amphotericin B (L-AmB) is advocated by guidelines for the treatment of azole-resistant aspergillosis infections. Preclinical research indicates that high-dose posaconazole (HD-POS) might be a feasible option provided that high drug exposure (i.e. POS serum through levels >3 mg/L) can be achieved and is safe.

Objectives

To describe our experience with the use of oral HD-POS as a treatment strategies for patients infected with pathogens with a POS MIC close to the clinical breakpoint.

Patients/Methods

We review evidence supporting the use of HD-POS and describe our experience on safety and efficacy in 16 patients. In addition, we describe the adverse events (AE) observed in 25 patients with POS concentrations at the higher end of the population distribution during treatment with the licensed dose.

Results

Sixteen patients were treated intentionally with HD-POS for voriconazole-resistant invasive aspergillosis (7/16), mucormycosis (4/16), salvage therapy for IA (4/16) and IA at a sanctuary site (spondylodiscitis) in 1. Grade 3-4 AEs were observed in 6 and all of them were considered at least possibly related. Grade 3-4 AEs were observed in 5 of the 25 patients with spontaneous high POS serum through levels considered at least possibly related using Naranjo scale.

Conclusions

HD-POS is a treatment option if strict monitoring for both exposure and for AE is possible.

INTRODUCTION

Invasive aspergillosis (IA) in patients with haematological malignancies is associated with a mortality of 20-30%. (1, 2) Triazole resistance is increasingly reported in different countries through culture-based surveillance studies, (3) and is associated with a much higher mortality of 50-88%. (4, 5) In 2015, a consensus meeting on the management of azole-resistant IA was organized (6) and liposomal-amphotericin B (L-AmB) was advocated as the preferred therapy but has obvious toxicity limitations and can only be administered intravenously. Treatment of IA has to be continued for a minimum of 6–12 weeks but occasionally much longer. (7) Other treatment options are therefore urgently needed. Phase II studies on new antifungals are just about to start and subsequent phase III studies typically take 3 or 4 years to complete. Therefore, these drugs will not provide a short term solution. Targeting high-exposure posaconazole (POS) may be a potential oral step-down treatment option for azole-resistant IA and other difficult-to-treat mould infections.

POS is approved in patients with haematological malignancies both for prophylaxis and treatment of refractory IA or when intolerance to first-line agents occurs. (8, 9) The agent is available as oral suspension, a delayed-release tablet and an intravenous formulation. Oral absorption of POS oral suspension is affected by food and gastric pH. In contrast, POS-tablets contains the active drug mixed with a pH-sensitive polymer (10) and this polymer releases the drug in the intestines, causing three-fold increased exposures compared to POS oral suspension. (11)

Therapeutic drug monitoring (TDM) has been widely implemented to assess therapeutic efficacy of POS oral suspension but its usefulness is in a state of flux following the introduction of the new POS formulations specifically in the setting of prophylaxis. (12-14) Current guidelines recommend a Ctrough concentration of ≥0.7 mg/L for prophylaxis and >1.0 mg/L for primary and >1.25 mg/L for salvage therapy, (15) although these concentrations were determined independent of the susceptibility of the infecting pathogen. (13)

These targets have been derived for susceptible pathogens and are not valid for pathogens with attenuated susceptibilities. A different approach is needed to optimize treatment in case of reduced susceptibility.

Preclinical research indicates that high-dose posaconazole (HD-POS) might be a feasible option provided that high drug exposure (i.e. POS serum through levels >3 mg/L) can be achieved and is safe. Hence, we argued that oral high-dose treatment strategies might be feasible to treat pathogens with relatively low MICs/MICs just above the clinical breakpoint (low-resistant). Human data on the treatment of pathogens with reduced susceptibility as well as safety of POS Ctrough concentrations of >3 mg/L are sparse.

Here, we review the evidence supporting the use of HD-POS and describe our experience on safety and efficacy in 16 patients. In addition, we describe the adverse events (AE) observed in 25 patients with POS concentrations at the higher end of the population distribution during treatment with the licensed dose.

Patients / Methods

We set out to explore safety of HD-POS and retrospectively collected clinical and laboratory data of patients from 2 Dutch academic medical centres (Erasmus University Medical Centre, Rotterdam and Radboud University Medical Centre, Nijmegen) in which POS Ctrough concentrations >3 mg/L had been documented in two different populations. All patients were in care by one of the authors of this paper. Data were extracted and reviewed by J.B. and A.S. Group 1 consisted of patients intentionally treated with HD-POS targeting POS Ctrough concentrations >3 mg/L and Group 2 were patients that reached POS Ctrough concentrations >3 mg/L with the licensed dose. We focused on AEs (related or unrelated to POS) described in the patient files and laboratory data. Data from these patients were reviewed for toxicities according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03. An AE was defined as unfavourable or unintended sign or symptom while the patient was treated with POS, whether or not the sign or symptom was related to POS. The Naranjo scale was used to determine for the assessment of causality of potential AE with POS. This is a questionnaire for determining a potential likelihood that an adverse drug reaction is actually linked to a drug. Probability is assigned using a scoring system with the following possible results: definite, probable, possible of doubtful. (16) Medians and 25th to 75th inter-quartile ranges were used for statistic descriptions. This type of research does not fall under the Dutch law of research on human subjects. However, to safeguard the privacy of the patients, the data were stored anonymously after data extraction.□

RESULTS

Group 1

Sixteen patients were treated intentionally with HD-POS for voriconazole-resistant IA (7/16), mucormycosis (4/16), salvage therapy for IA (4/16) and IA at a sanctuary site (spondylodiscitis) in 1. The median POS dose given was 600 (IQR 400,750) mg daily when the POS Ctrough concentrations of >3 mg/L was reached after a median of 8 (IQR 6,40) days. Ten patients had significantly higher Ctrough concentration (above 4 mg/L) and 6 patients had Ctrough concentrations between 3.0 and 4.0 mg/L and on average patients had these concentrations for a median 76 days (IQR 20,162). Thirteen patients received POS-tablet, 1 patient posaconazole-oral suspension (POS-OS) and 2 patients a combina-

tion of formulations. AEs are described in table 1. Grade 3-4 AEs were observed in 6 patients and all of them were considered at least possibly related using Naranjo scale. In 3 out of16 patients the treatment was stopped following an AE: arterial hypertension (grade 2), QTc prolongation, cardiac troponin T increased and left ventricular failure (grade 3) and leukocytopenia (grade 4).

Efficacy

Of the 7 patients with azole-resistant IA treated with HD-POS, 4 survived while 3 died from their underlying disease but unrelated to the IA. In 2 patients HD-POS was used as salvage therapy. One patient with IA caused by *A. terreus* was treated with HD-POS because serum galactomannan levels increased under conventional dosage which is a predictor of poor outcome (table 3). All patients with mucormycosis survived.

Group 2

This group consisted of 25 patients. The median POS Ctrough concentration was 4.3 mg/L (IQR 3.5-6.0). 19, 5 and 1 patient received POS-tablet, POS-OS and the IV formulation respectively. Posaconazole was given to 18 and 7 patients for prophylaxis and treatment, respectively. All observed AEs are described in table 2. The most frequently

Adverse event	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Diarrhoea	1				
Nausea		1			
Vomiting	3				
Increased hepatic enzymes	4	1	1(3)	2(5|7)	
Cardiac troponin T increased			1(6)		
Electrocardiogram QTc corrected interval prolonged	1	1	1(6)		
Leukopenia				1(4)	
Hypokalaemia	3	3			
Hyperkalaemia	1				
Headache		1			
Delirium	1		1(2)		
Alopecia	1				
Hypertension		2			
Heart failure			1(5)		
Rash	1				

Table 1: Adverse events of 16 patients receiving intentionally HD-POS graded accordingly to the Common Terminology Criteria for Adverse Events (version 4.03). Digits refer to the number of patients in whom these AEs have been documented. Prolongation in the QTc interval was assessed by comparing electrocardiograms obtained at baseline and during HD-POS treatment, if available. (*) Naranjo adverse drug reaction probability scale: >9: definite, 5 to 8: probable, 1-4: possible. -3 to 0: doubtful.

observed AE were hypokalaemia in 8 patients and neurological in 6 patients (headache, convulsions). Grade 3-4 AEs were observed in 5 and all of them were considered at least possibly related using Naranjo scale. In 8 of the 25 patients the dosage was reduced. Follow-up Ctrough concentrations were between 1.1 and 4.3 mg/L after dosage reduction.

DISCUSSION

Little is known about the toxicity of patients attaining high POS Ctrough of >3 mg/L. The upper boundary level of average POS serum concentrations of 3.75 mg/L is set by the European Medicines Agency based on experience with the POS-OS and preclinical toxicology findings (17). In this study, we reviewed the safety and tolerability of HD-POS. In both group 1 and group 2, three patients were seen with a combination of hypertension and hypokalaemia that required antihypertensive therapy and potassium supplementation. The most striking case was a child treated with POS, L-AmB and micafungin for a proven aspergillosis following surgical removal of *Aspergillus* lesions in the

	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Adverse event					
Diarrhoea	4				
Nausea	4				
Vomiting	2				
Increased hepatic enzymes	2	3	1(4)		
Electrocardiogram QT corrected interval prolonged	2	1			
GGT increased		1			
Anorexia	5	1			
Hyponatremia	2		1(1)		
Hypokalaemia	7	1			
Headache	5				
Seizure		1			
Alopecia	2				
Hypertension		2	1(7)	1(4)	
Hypotension					1(#)(7)
Rash	3				

Table 2: Adverse events of 25 patients receiving POS with high spontaneous concentration graded accordingly to the Common Terminology Criteria for Adverse Events(version 4.03). Digits refer to the number of patients in whom these AEs have been documented. (*)These grade 3 or 4 AE were considered at least possible related to POS.(#) Refractory shock, rapidly fatal. Distributive shock most likely according to treating physician.(*) Naranjo adverse drug reaction probability scale: >9: definite, 5 to 8: probable, 1-4: possible. -3 to 0: doubtful.

Patient	Age (years)	Underlying disease	IFD, classification	Reason HD-POS	Sample with culture	Aspergillus PCR result	MIC(mg/L)*				POS concentration: calculated target		Outcome
							ITZ	VCZ	POS	ISA	Highest C	Calculated Target	
1	69	Mixed dust pneumoconiosis	CPA	Resistant strain	Sputum: *A. fumigatus*	TR_{46}/ Y121F/ T289A	>16	8	1	4	3.8	6.18-6.66	Alive
2	51	AML, AlloTx	IPA, probable	Resistant strain	No positive culture	Y121F/ T289A in BAL					6.1		Dead
3	18	ALL	IPA, proven (cerebral)	Resistant strain	Sputum: *A. fumigatus*	TR_{34}/L98H	16	8	2	8	6	>10	Alive
4	46	SOT (kidney), PTLD	IPA, probable	Resistant strain	BAL: *A. fumigatus*	TR_{34}/L98H	>16	4	0.5	8	0.2[b]	3.09-3.33	Dead
5	69	AML	IPA, probable	Resistant strain	No positive culture	TR_{34}/L98H in BAL					4		Dead
6	61	No relevant	CPA	Resistant strain	BAL: *A. fumigatus*		>16	8	1	8	6.6	6.18-6.66	Alive
7	32	SOT (lung)	Pulmonary mucormycosis, proven	Mucormycosis	Lung: *Rhizopus* species		1	8	0.25	1	3.8	1.44-1.55	Alive
8	17	ALL	IPA, probable	Mixed infection (R/S)	BAL: *A. fumigatus* R and S		>16	4	0.5	8	5.6	3.09-3.33	Alive
9	50	AML, AlloTx	Mucormycosis, probable	Mucormycosis	No positive culture						5.2		Alive
10	58	SLE with pancytopenia	Mucormycosis, proven	Mucormycosis	Liver biopsy: microscopy: hyphy. No positive culture. Spleen biopsy PCR positive	*PCR: Rizomucor pussilus*					5.0		Alive
11	67	DM type II	Mucormycosis, probable (skin)	Mucormycosis	Tissue sample wound: *Rhizopus oryzae*		0.25	8	0.25	1	3.5	1.44-1.55	Alive
12	2	ALL	Mucormycosis, proven	Mucormycosis	Multiple skin biopsies: *Lichtheimia corymbifera*		0.5	16	0.5	>16	6.6	3.09-3.33	Alive

Patient	Age (years)	Underlying disease	IFD, classification	Reason HD-POS	Sample with culture	Aspergillus PCR result	MIC(mg/L)*				POS concentration: calculated target		Outcome
13	50	No relevant	IA, proven	Sanctionary site infection	Spinal biopsy: *A. fumigatus*		0.25[a]	0.25[a]	0.063[a]	0.5[a]	3.6		Alive
14	68	AML	IPA, probable	Salvage	No positive culture						3.8		Dead
15	65	AML	IPA, probable	Salvage	Sputum: *A. nidulans*	Wild-type *A. fumigatus* in BAL	0.25	0.25	0.25	0.5	3.1	1.44-1.55	Dead
16	8	ALL	IPA, proven	Salvage *A. terreus*	Lobectomy, lung tissue: *A. terreus*	Lung biopsy: *Aspergillus* Species	0.125	1	0.031	1	4.7		Alive

Table 3: Underlying condition, IFD, *A. fumigatus* genotype and phenotype, and outcome in 16 patients treated with high-dose posaconazole (HD-POS). *MIC was determined according to the EUCAST method for susceptibility testing of moulds (version 9.2). Patients were classified following the revised definitions of the European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG). (42)

[a]MIC was determined according to the CLSI method for susceptibility testing of moulds(M38-A2); [b]This patient was included because the patient was treated with POS 400mg BID despite the low Ctrough level.

Abbreviations: C concentration, HD-POS high-dose posaconazole, SOT solid organ transplantation, AlloTx allogeneic stem cell transplant, R: resistant, S: Susceptible, IPA Invasive pulmonary aspergillosis, CPA chronic pulmonary aspergillosis, IA invasive aspergillosis, IFD invasive fungal diseases, PTLD post-transplant lymphoproliferative disease, ITZ Itraconazole, VCZ voriconazole, POS posaconazole and ISA isavuconazole. Calculated target Ctrough based on the MIC is taken from Seyedmousavi et al. (28)

spleen, left lung and right kidney. This patient developed several hypertensive crises and developed hypokalaemia for which oral supplementation was needed. 8 months after POS treatment, the patient died due to a vasopressor refractory shock. During these hypertensive crises, aldosterone could not be measured (<50 pmol/L) and renine was within normal range. In retrospect, POS may have caused the hypertension, and hypokalaemia. Recently, a case of POS induced heart failure, hypertension and hypokalaemia was described with low renin and aldosterone levels. The inhibition of the enzyme 11-beta-hydroxysteroid dehydrogenase 2 is suggested as the potential mechanism causing apparent mineralocorticoid excess. (18-20) This enzyme is homeostatic regulator and damps mineralocorticoid activity by converting cortisol to cortisone.

The AE of HD-POS observed in this study are in line with previous reports of AE due to POS. A phase III study assessing PK and safety of POS-tablet demonstrated that nausea and diarrhoea were the most common treatment-related AEs leading to POS discontinuation in 2% and 1%, respectively. (21) Only 9 patients (10%) in this study attained an average Ctrough concentration between 2.5 and 3.75 mg/L and six patients (3%) reached Ctrough concentrations ≥3.75 mg/L. No increase of AEs in patients with higher POS serum concentrations was observed but the study was not powered to detect such a relation. Very recently, PK and safety results from a phase 3 study of IV POS in patients at risk for invasive fungal disease were published. Six percent of the patients had a steady-state concentration between >2.5 and ≤3.65 mg/L without signs of toxicity. (22) In a retrospective analysis of 64 patients receiving POS-tablet as prophylaxis, median POS steady state concentrations of 1.67 mg/L (0.52-3.83 mg/L) were documented. In 21% of the patients a QT_c prolongation was observed and the median steady state concentration was 2.04 mg/L. (23) In a single-centre study, 343 courses of POS prophylaxis (IV or tablet) were assessed for safety and effectiveness. 20% of these patients developed liver injury, mostly hyperbilirubinemia but this is often multifactorial. More importantly, grade 3-4 elevations in hepatic enzymes were only observed in 2% of the patients without pre-existing liver injury with mostly spontaneous resolution despite treatment continuation. (24) Thus, in the current literature, information about the toxicity of high POS serum concentrations is limited but no increase in the number of AEs was observed in patients with higher than average serum concentrations.

Azole-resistant IA

The large majority of azole-resistant *A. fumigatus* isolates harbour TR_{34}/L98H or TR_{46}/Y121F/T289A mutations in the *cyp*51A gene, (25, 26) encoding the cytochrome p450 sterol 14α-demethylase, the target of azoles. *A. fumigatus* isolates carrying resistance associated mutations have high minimal inhibitory concentrations (MICs) for itraconazole and/or voriconazole as well as isavuconazole. (27) The MIC of POS often remains close to the susceptible population (i.e. MIC ≤0.5 to 1 mg/L). (28) MIC levels of POS

>0.25 mg/L are considered resistant according to the EUCAST breakpoint, but this is based on population susceptibility and on concentrations achieved with the POS-OS at licensed dose. Indeed, drug exposure with POS-OS will marginally cover the *A. fumigatus* wild-type population, let alone low-level POS-resistant isolates. Higher exposures can be achieved with the newer formulations. (13) The pharmacodynamic-pharmacokinetic (PK-PD) relationships of POS have been studied *in vivo*. A murine model of IA indicated that low-level POS-resistant isolates can be treated when the POS exposure is increased. Two *in vivo* studies demonstrated that POS retains efficacy against *A. fumigatus* isolates with POS-MIC of 0.5 mg/L as long as POS exposure is sufficiently high. (29, 30) Based on these experiments, the required POS exposure (area-under-the concentration time curve (AUC)) in patients can be calculated for isolates with an increased MIC. The probability of target attainment for treatment of IA using standard dosing of POS-tablet is estimated to be ~80 % for isolates with POS-MIC of 0.25 mg/L and >90% for isolates with a POS-MIC of 0.125 mg/L. (28) The probability of target attainment for a POS-MIC of 0.5 mg/L was 24% and for a POS-MIC ≥ 0.5 mg/L it was 0%.

As determination of the AUC requires multiple sampling moments, and this AUC is linear correlated to Ctrough concentrations, quite often the Ctrough concentrations are used in daily practice as surrogate markers. (13, 28) Monte Carlo simulations estimated that the POS Ctrough concentrations needed to be 1.44-1.55 mg/L for isolates with a POS-MIC of 0.25 mg/L and 3.09-3.33 mg/L for isolates with a POS-MIC of 0.5 mg/L. (28)

As the aforementioned *in vivo* experiments indicated that *A. fumigatus* with a POS-MIC of 0.5 mg/L can be treated with elevated POS dosing, we hypothesized that targeting high exposure with HD-POS is an oral step-down treatment option for azole-resistant IA. Although clinical evidence supporting HD-POS has not been described, preclinical animal studies and experience in veterinary medicine provided proof-of-principle for its efficiency. (28, 29)

Mucormycosis

Limited *in vivo* models are available that assess POS for the treatment of mucormycosis. A neutropenic mouse model indicated similar pharmacodynamics for mucormycosis compared to *A. fumigatus* infections. An AUC/MIC of 87 was needed to treat *Rhizopus oryzae* infection, which was comparable to the target needed for IA (AUC/MIC of 76). (31) Efficacy of POS showed a dose-response relationship in another *in vivo* model of experimental mucormycosis in which a dose of 100mg/kg/day showed significant reduction of mortality of *Lichtheimia corymbifera* infection. (32) Similar dose-response relationships were seen for *Mucor* species and *R. oryzae*. (33, 34) Compared to *A. fumigatus* isolates, the MICs of Mucorales are often higher with a geometric mean CLSI MIC of 0.39 mg/L (35) and an epidemiological cut-off value of 1 mg/L for *L. corymbifera*, *R. oryzae*, and *R. microspores* and 4 mg/L for *M. circinelloides* (Figure 1). (36) Furthermore, the

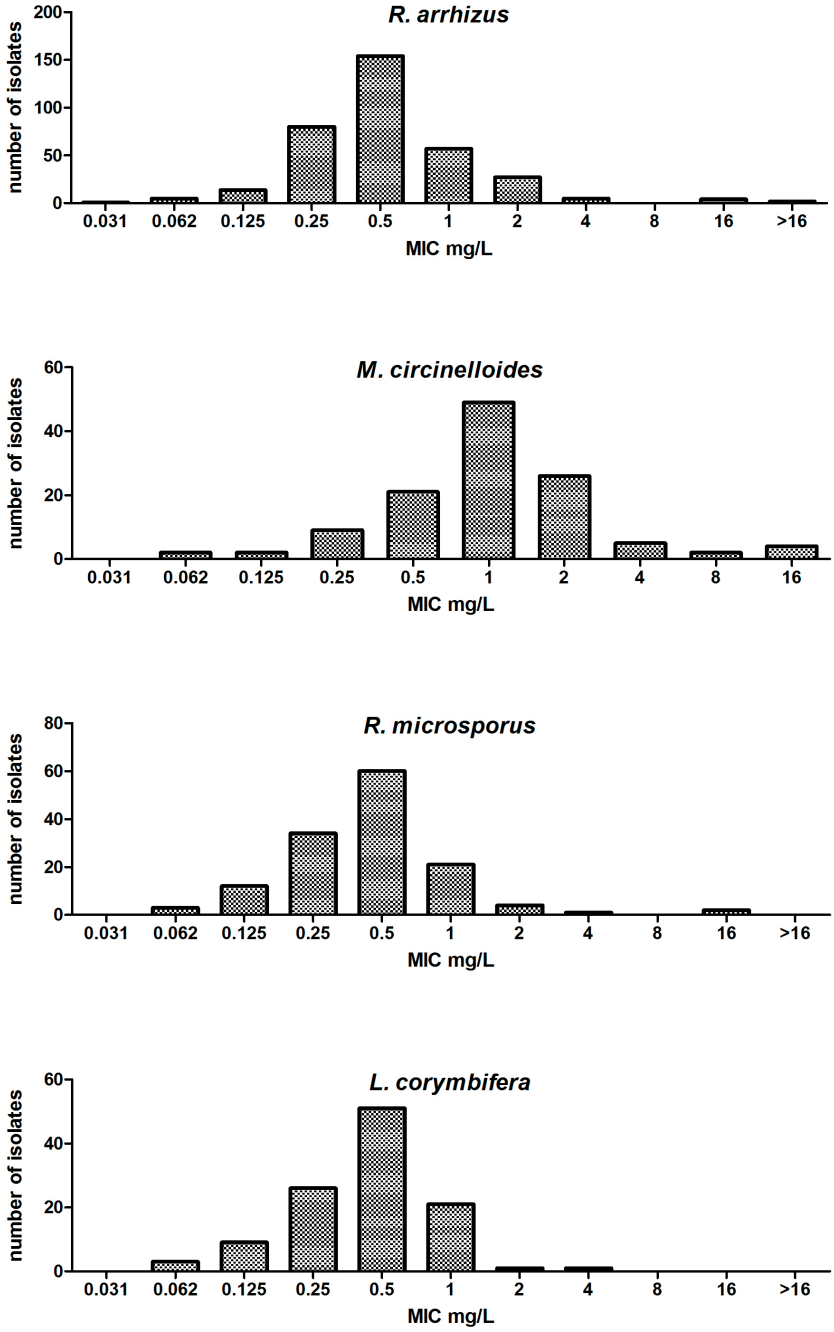

Figure 1: Posaconazole MIC distributions of most common Mucorales species: *Rhizopus oryzae*, *Mucor circinelloides*, *Rhizopus microspores and Lichtheimia corymbifera*
MICs were extracted from Espinel-Ingroff et al. (36) MICs were determined according to the CLSI method for susceptibility testing of molds (M38-A2).

EUCAST MICs for Mucorales are higher than CLSI MICs for most species. (37) Taken into account the similar target AUC/MIC for Mucorales as *A. fumigatus*, but higher MICs for Mucorales isolates compared to *A. fumigatus*, it seems reasonable to pursue higher than normal POS serum concentrations for the treatment of mucormycosis as long as this is not associated with toxicity. (13)

POS-OS has been used as salvage therapy for mucormycosis with a success rate of approximately 60-80%. (38) A recently published matched-paired analysis assessed the clinical effectiveness and safety of POS tablets and intravenous formulation in comparison with amphotericin B as first-line treatment and with POS-OS as salvage treatment for invasive mucormycosis. POS tablets and intravenous formulation were effective in terms of treatment response and associated mortality. However, these observations should be interpreted with caution given the small sample size in this study (43). Clinical data on PK/PD are lacking due to limited susceptibility data from clinical studies. (38)

Dosing and TDM

The pharmacokinetics of posaconazole tablets are best described by a one-compartment pharmacokinetic model with sequential zero-order and first-order absorption and a first-order disposition from the central compartment. Recently, several covariates were identified influencing bioavailability (like disease state, body weight, formulation), adsorption rate (food status) and clearance (dosing regimen) of POS tablets. Only body weight was considered clinically relevant. (39) Knowledge on the PK of POS helps to identify the optimal dose when targeting high exposure. Subsequently, an infrastructure is needed where one can quickly assess drug concentrations to deploy an adaptive approach in terms of dosing. With the new formulations of POS a loading dose is given, which enables early assessment, typically by day 3, of POS concentrations. Follow-up samples are measured again before the 5th dose of every changed dosage.

The pharmacokinetics described above translate into an expected doubling of the Ctrough concentration when the dose of POS-tablet or IV formulation is doubled. For example, when the Cthrough concentration is 1.5 mg/L, increasing the dose from 300 mg once daily to 300mg twice daily can be expected to lead to a serum concentration of 3 mg/L. For safety reasons, we advise to increase the dose with no more than 200 mg per step.

Inhibitory potential of HD-POS

POS is a strong CYP3A4 inhibitor and the clinician should therefore also remain vigilant for drug interactions. In our case series, we had two patients with significant interactions. Toxicity of HD-POS in combination with vincristine was seen in a child with ALL, resulting in hepatotoxicity, convulsions and hypertension which might be attributed to

the inhibition of CYP3A4 as well as P-gp resulting in increased levels of vincristine. (40) Another allogeneic stem cell transplant patient developed IA despite prophylaxis with voriconazole. Treatment with L-AmB was started but switched to HD-POS for progressive renal impairment. POS Ctrough concentration was 5.2 mg/L. After the patient was treated with panobinostat, a histone deacetylase (HDAC) inhibitor, grade 4 leukopenia developed. After 4 weeks of persisting grade 4 leukopenia, POS treatment was stopped as presumed culprit and leukopenia improved. This interaction could have been predicted based on the interaction of panobinostat with ketoconazole where panobinostat maximum serum concentrations were increased by an average of 1.6-fold. (41)

Safety monitoring for HD-POS

We propose that the following safety measures are taken if HD-POS is used as a treatment strategy. At least the following laboratory tests should be performed twice weekly during the first 2 weeks and as long as the POS dosage is being increased: electrolytes, renal clearance, haemoglobin, leukocyte differentiation, thrombocytes and liver enzymes. Posaconazole, may cause QT prolongation. Therefore, an ECG should be recorded before the start of HD-POS as well as during treatment. If no lab abnormalities possibly related to POS are observed the monitoring interval can be increased.

In conclusion, registration of new antifungals with efficacy against azole-resistant *A. fumigatus* is expected to take several more years. Therefore, targeting high serum concentrations of POS using the tablet or IV formulation is, in our point of view, a possible step-down option in patients with azole-resistant IA as long as the POS-MIC is <1 mg/L and for patients treated for mucormycosis with L-AmB. It should only be used when close monitoring for AE is implemented as described above in conjunction with TDM and when the benefits are likely to outweigh the risks.

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Author Contributions

A.F.A.D.S. and J.B.B. collected the clinical data. A.F.A.D.S., J.B.B., R.J.B. and B.J.A.R. analyzed the data. A.F.A.D.S., J.B.B., R.J.B. and B.J.A.R. wrote the initial draft. All authors critically revised the initial draft and final manuscript.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this type of research does not fall under the Dutch law of research on human subjects.

Transparency declarations

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Chapter 5

Outpatient Parenteral Antifungal Therapy (OPAT) for Invasive Fungal Infections with intermittent dosing of Liposomal Amphotericin B.

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Alle wegen leiden naar Rome maar de weg tussen Antwerpen en Rotterdam is bezaaid met ontelbare obstakels wegens een falend openbaar vervoer. Brompton-gerelateerd reisadvies kan worden bekomen bij Rijnders & Schauwvlieghe Travel Agency.

ABSTRACT

Background

Triazole resistant *A. fumigatus* has been documented in many parts of the world. In the Netherlands, incidence is now above 10% and results in the need for long-term parenteral therapy with liposomal amphotericin B (LAmB). The long terminal half-life of LAmB suggests that intermittent dosing could be effective, making the application of outpatient antifungal therapy (OPAT) possible. Here, we report our experience with the use of OPAT for Invasive Fungal Infections (IFI).

Methods

All adult patients treated with LAmB with a 2 or 3 times weekly administration via the outpatient departments in four academic tertiary care centres in the Netherlands and Belgium since January 2010 were included in our analysis. Patient characteristics were collected, as well as information about diagnostics, therapy dose and duration, toxicity, treatment history and outcome of the IFI.

Results

In total, 18 patients were included. The most frequently used regimen (67%) was 5mg/kg 3 times weekly. A partial response to the daily treatment prior to discharge was confirmed by CT-scan in 17 (94%) of patients. A favourable outcome was achieved in 13 (72%) patients. Decrease in renal function occurred in 10 (56%) cases but was reversible in all and was treatment limiting in 1 patient only. 100-day mortality and 1-year mortality after initiation of OPAT were 0% and 6%, respectively.

Conclusions

In a selected population, and after confirmation of initial response to treatment, our data support the use of OPAT with LAmB for treatment of IFI in an intermittent dosing regimen.

BACKGROUND

Invasive Fungal Infections (IFI) are often life-threatening and occur predominantly in immunocompromised patients. After surviving the initial phase of infection, prolonged treatment with an antifungal agent is often necessary to ensure complete resolution (1, 2). Unfortunately, the different antifungal drugs in the current medical armamentarium all have shortcomings when used for a prolonged period of time (3). For invasive aspergillosis (IA) voriconazole became the first-choice treatment after an improved survival was documented over conventional amphotericin B (cAmB). Furthermore, voriconazole has a favourable adverse event profile compared to conventional formulations of amphotericin B and it is rarely associated with renal toxicity (4, 5). Nonetheless, no direct comparison between voriconazole and the more well-tolerated liposomal amphotericin B (LAmB) has been made. In recent years, increasing rates of triazole resistant *Aspergillus* fumigatus in particular in Europe but also in other continents have become a major concern (6-10). This has led to a renewed incentive to reconsider therapeutic strategies using LAmB (11, 12). For many IFI caused by non-Aspergillus fungi, e.g. *Mucorales* spp., LAmB already is the preferred first-line treatment (13, 14). Therefore, treatment with LAmB is increasingly indicated and sometimes even the last resort in the management of invasive fungal disease.

LAmB is solely administered in an intravenous formulation. Both safety concerns and logistical reasons prevent dismission from the hospital during intravenous treatment; however, often the treatment duration is long and exceeds the period of necessity of hospitalisation for clinical reasons (1, 2). The practical limitations of daily intravenous treatment are evident. Reduction of duration of hospital stay would be favourable when considering both patient quality of life as well as economic costs. Furthermore, continued daily intravenous administration will lead to high cumulative dosages, associated with a higher rate of adverse events. As an alternative, we have started to apply Outpatient Antifungal Therapy (OPAT) with LAmB. OPAT has been implemented successfully in the past with various antibiotics. In bacterial infections, increasing antimicrobial resistance rates have made prolonged intravenous treatment with reserve antibiotics necessary. For example, the increasing rate of Methicillin Resistant *Staphylococcus aureus* has been an important reason to apply prolonged OPAT with vancomycin (15-17). With LAmB, outpatient use has recently been implemented in a prophylactic setting (18).

Two recent reviews of the pharmacokinetic properties of LAmB strengthen the hypothesis that LAmB can effectively be applied as OPAT (19, 20). LAmB has a relatively short elimination half-life of 7 hours shortly after initiation of therapy, which increases to over 100 hours after prolonged use. This phenomenon is attributed to accumulation in tissues and slow redistribution (21, 22). When these pharmacokinetic properties of

LAmB are taken into account, (23-25), it can be expected that a therapeutic concentration can be attained is a less frequent dosing scheme. Moreover, it may be possible to (partially) avoid nephrotoxicity if the total dose of LAmB is spread over multiple days (25, 26). Nephrotoxicity however remains an important caveat in the application of OPAT with LAmB, as mentioned in pharmacological review papers and in previous experimental experience (19, 20, 22, 27).

For those in need of prolonged antifungal treatment, step-down therapy to intermittent dosing in the context of outpatient treatment could offer similar efficacy with the potential of improved safety. An intermittent dosing strategy is occasionally applied in several hospitals in the Netherlands and Belgium. In this study, we are introducing the concept of treatment of IFI with intermittent LAmB dosing as OPAT.

METHODS

Study Setting and patient population

A multi-centre retrospective cohort study was conducted within the Netherlands and Belgium. Hospitals that participate in the Dutch-Belgian Mycoses study group (DB-MSG) (28), a consortium committed to the clinical research of IFI, were sent an inquiry about their experience in the application of OPAT with LAmB in the past 10 years. Of the 11 medical centers that participate in the DB-MSG, four responded that they had applied OPAT with LAmB in recent years. OPAT was applied at the home of the patient or within the hospital outpatient department. All adult patients treated with LAmB with a less frequently than daily administration via the outpatient departments of Leiden University Medical Center, Erasmus MC Rotterdam, Radboud University Medical Center Nijmegen, and the University Hospitals Leuven since January 2012 were included. These centres are all tertiary care university hospitals and engaged in extensive solid organ and hematopoietic stem cell transplantation programs.

Study protocols and definitions

No uniform protocols for the start of intermittent therapy with LAmB were present. Typically for *Aspergillus* disease, a 3 mg/kg dose was started. For *Mucor* species a typical dose was between 5-10 mg/kg. The choice to start treating with intermittent therapy with LAmB was made according to the clinical judgement of the treating physician usually based on imaging and clinical course. Patients that were started on OPAT with LAmB were closely monitored for the occurrence of nephrotoxicity and most patients received the drugs in the outpatient department of the hospital. In the first month, all patients had at least a weekly monitoring of electrolyte and kidney function. In the subsequent weeks, monitoring occurred at least once every two weeks.

Nephrotoxicity was defined as a >1.5 times increase of baseline serum creatinine levels resulting in an eGFR of less than 40 ml/min/1.73 m^2 during treatment or as electrolyte disorders suspected to be the result of renal damage and requiring cessation of treatment with LAmB at the discretion of the treating physician. Resolution of IFI was defined as clinically observed absence of complaints that are likely to be caused by IFI in combination with findings concordant with resolution of IFI on high-resolution CT-scan and the absence of the need to restart antifungal therapy within 6 months.

Data collection

At the participating sites, lists of patients that received LAmB as an outpatient were provided by the hospital pharmacy. Based on these lists, the electronic medical records were examined to ensure eligibility for inclusion in our study. The only inclusion criterion was at least 2 weeks of intermittent treatment outside of the hospital with LAmB for an invasive fungal infection meeting the diagnostic criteria of the revised (2008) EORTC/MSG definitions for invasive fungal disease (29).

After retrieval of all relevant information, the data of all participants was pseudonymized. Patient characteristics including age, diagnosis of immunocompromising disease, diagnosis of IFI, comorbidity and immune status were collected, as well as information about performed diagnostics, dosage of therapy, duration of therapy, treatment history, switch of antifungal therapy, renal function and outcome of the IFI. The latter three variables were considered the primary study outcomes to assess the safety and efficacy of this strategy. IFI were classified according to the 2008 revised European Organisation for Research and Treatment of Cancer – Mycoses Study Group criteria for the classification of IFI (29).

Analyses

Descriptive statistics of clinical variables of patients were calculated using the complete dataset. Kaplan Meier curves of survival during OPAT with LAmB were constructed. The analyses were performed using STATA v 16 (Statacorp, College Station, Texas, USA).

Ethics

The study was reviewed by the institutional review board of the LUMC Leiden in the Netherlands, which confirmed that the study did not fall under the Dutch law on research on human subjects. The institutional review board from UZ/KU Leuven in Belgium approved the study. Data were processed after pseudonymization by the local investigators and in accordance with Personal Data Protection Acts of the respective countries.

RESULTS

Between January 1st 2010 and September 1st 2018, a total of 18 adult patients received LAmB as an outpatient in a dosing frequency of two or three times a week. Triazole resistance, demonstrated by either PCR or culture, has been the most common reason (in 10 cases) to choose treatment with LAmB instead of voriconazole in the patients with Invasive Aspergillosis. Of all patients, nine (50%) were male and median age was 60 years. Fourteen patients (78%) had a haematological malignancy as underlying predisposing disease. Other underlying diseases were Chronic obstructive Pulmonary Disease (COPD), Sickle Cell disease and Chronic Granulomatous Disease (CGD). Suspected causative agents of IFI were *Aspergillus* spp. (12 patients), *Mucorales* spp. (3 patients), *Fusarium* spp. (2 patients) and a combination of both Aspergillus and Mucor (1 patient). Table 1 summarizes the descriptive characteristics of the study cohort. A response to treatment prior to discharge and start of OPAT with LAmB was confirmed by CT-scan in 17 patients. For the remaining patient, clinical improvement had been the reason to proceed with OPAT. Patients switched from daily treatment as an inpatient to intermittent OPAT with LAmB after a median of 56 days (range 14-193 days). Median dosage of liposomal amphotericin B was 3 mg/kg, administered three times each week. Some patients switched drug dosage and/or frequency as detailed in the legend. None of the patients received combination therapy. Resolution of infection was finally achieved in 13 patients. The remaining patients were readmitted to the hospital, switched to another antifungal, died or were lost to follow-up.

Nephrotoxicity during OPAT occurred in 10 cases, of which in only one case treatment needed to be switched to another antifungal agent (posaconazole, after establishing intermediate sensitivity).

All patients in our dataset had normalised renal functions after decreasing of dosage or cessation of LAmB therapy. Severe hypokalaemia (less than 2.5 mmol/litre) was not observed during treatment with LAmB in an intermittent scheme. No intravenous or oral substitution of potassium was has been applied.

For the remaining cases, the treating physician opted for a dose reduction (four cases) or, after establishing a sufficient treatment response, for the cessation of antifungal therapy (five cases). The 100-day mortality and 1-year mortality were 0 and 1 patients out of 18 respectively. All-cause mortality until the end of follow-up was 39% but was related to the underlying immunocompromising disease. In all cases treated for invasive aspergillosis, the reason to treat with LAmB was triazole resistance (demonstrated in 10 patients, presumed in 3 patients). Readmission to the hospital was necessary due to factors related to the infection (3 patients) or to LAmB-related nephrotoxicity (1 patient). Figure 1a shows the survival rates of all patients in a Kaplan Meier analysis since start of OPAT. Figure 1b shows the time until resolution of infection. Figure 1c shows the time until nephrotoxicity occurred during intermittent treatment.

Total number of patients	18
Patient Characteristics	
Sex, male (%)	9 (50)
Age, median (range)	60 (18-78)
Underlying predisposing disease, number of pts. (%)	
ALL	6 (33)
AML/MDS-RAEB2	4 (22)
CLL	3 (17)
COPD	2 (11)
Aplastic Anemia	1 (6)
CGD	1 (6)
Sickle Cell Disease	1 (6)
Prior allogeneic HSCT for any underlying disease	8 (44)
Invasive Fungal Infection, number of pts. (%)*	
Aspergillosis	13 (72)
Mucormycosis	3 (17)
Fusariosis	2 (11)
Cryptococcosis	1 (6)
Reason to treat Invasive Aspergillosis with LAmB, Number of patients (% of patients with IA)	
Triazole resistance identified with culture or PCR	10 (77)
Resistance presumed because IA occurred despite adequate prophylaxis with a triazole	2 (15)
Resistance presumed because IA showed progression despite adequate treatment with a triazole	1 (8)
Treatment	
Dosage in mg/kg and frequency in times/week†, number of patients treated with the regimen at any point	
2 mg/kg 3 times/week	1
3 mg/kg 2 times/week	1
3 mg/kg 3 times/week	12
5 mg/kg 3 times/week	2
6 mg/kg 3 times/week	5
10 mg/kg 2 times/week	2
Response to treatment confirmed by CT before start of intermittent therapy, number of pts (%)	17 (94)
Number of days between date of diagnosis and start of intermittent therapy, median number of days (range)	56 (14-193)
Nephrotoxicity^, number of patients (%)	
Occurrence of nephrotoxicity at some point during intermittent LAmB treatment Of which	10 (56)
- resulting in switch to other antifungal agent	1 (10)
- resulting in cessation of antifungal treatment (because of concurrent sufficient clinical and radiological response to treatment)	4 (40)
- resulting in dose or frequency reduction–	5 (50)

Table 1: Patient characteristics
Legend: On next page

Legend: LAmB denotes liposomal amphotericin B, ALL Acute Lymphoid Leukaemia, AML Acute Myeloid Leukaemia, MDS-RAEB2 Myelodysplastic Syndrome with Refractory Anaemia with Excess Blasts-2, CLL Chronic Lymphatic Leukaemia, HSCT hematopoietic stem cell transplantation, CT Computed tomography, COPD Chronic Obstructive Pulmonary Disease, PCR Polymerase Chain Reaction, CGD chronic granulomatous disease, IA invasive Aspergillosis. * Numbers add up to more than 100% due to one patient suffering from an infection caused by both Mucor and Aspergillus. ^Nephrotoxicity defined as either serious electrolyte disturbances necessitating treatment cessation at the discretion of the treating clinician or at least 50% increase of creatinine levels resulting in a eGFR of less than 40 ml/min. †Numbers add up to more than 100% because of 5 patients with dose alterations during the study period. ~ Dose reductions were as follows: 2 patients treated with 6 mg/kg 3 times weekly and 1 patient treated with 5 mg/kg 3 times/week were switched to 3 mg/kg 3 times weekly. Of 2 patients treated with 3mg/kg 3 times/week, one was switched to 3mg/kg 2 times/week and 1 patient was switched to 2mg/kg 3 times/week. Kidney function normalised in all 5 patients. ‡ Resolution of infection defined as clinically observed absence of complaints that are likely to be caused by Invasive Fungal Infection in combination with clinically irrelevant or absent abnormalities concordant with Invasive Fungal Infection on High-resolution CT-scan.

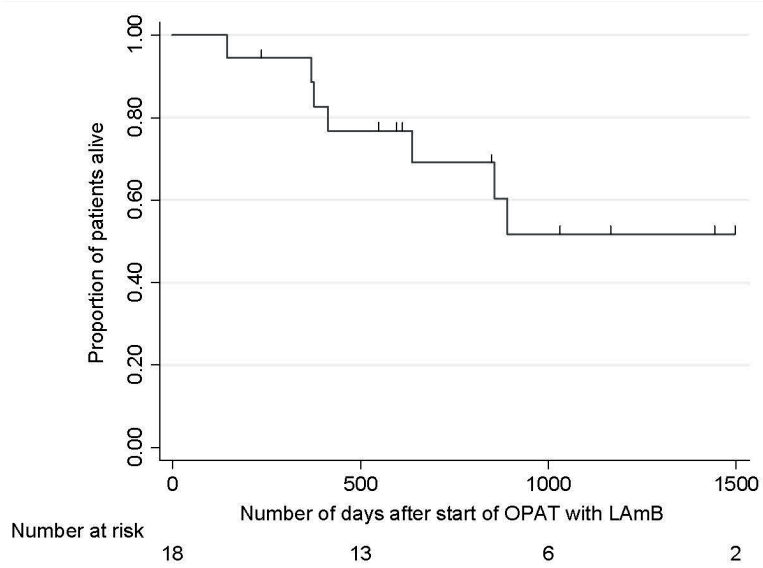

Figure 1A: Overall survival from start of intermittent treatment
Legend: OPAT denotes outpatient antifungal therapy, LAmB liposomal amphotericin B. Censored cases were lost to follow up.

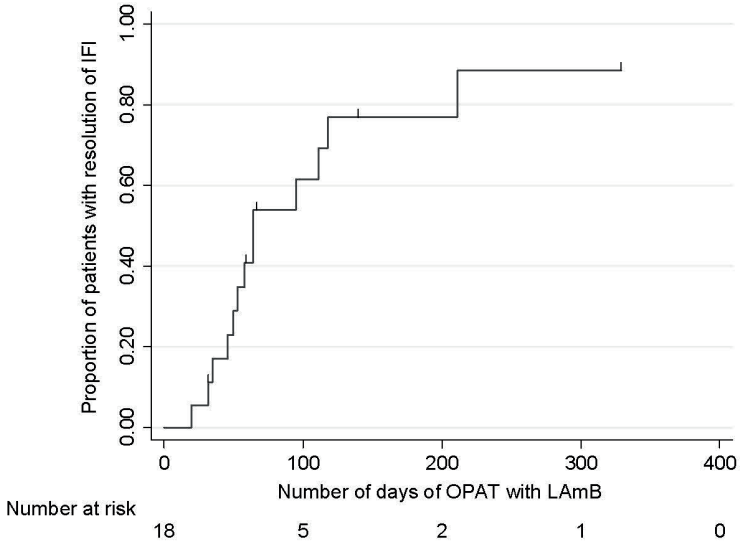

Figure 1B: Time to resolution of IFI after start of intermittent therapy.
Legend: IFI denotes invasive fungal infection, OPAT denotes outpatient antifungal therapy, LAmB liposomal amphotericin B. Censored cases stopped intermittent treatment before resolution of infection. Resolution of IFI was defined as clinically observed absence of complaints that are likely to be caused by IFI in combination with findings concordant with resolution of IFI on High-resolution CT-scan.

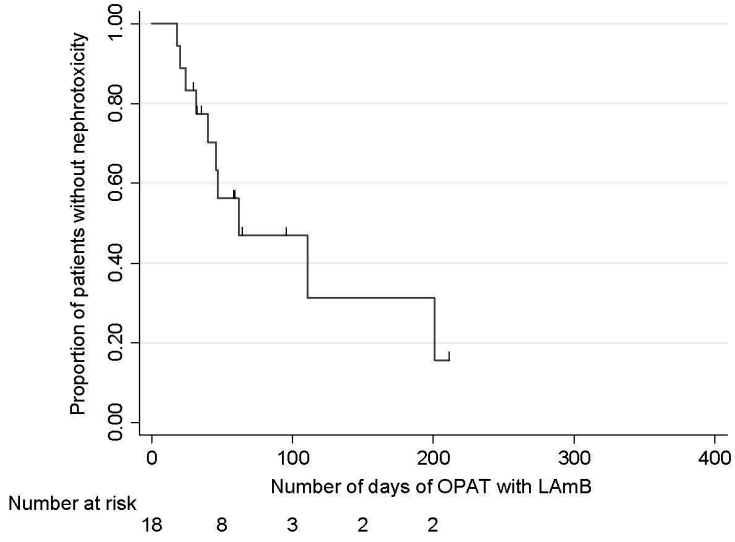

Figure 1C: Occurrence of nephrotoxicity from start of intermittent treatment
Legend: OPAT denotes outpatient antifungal therapy, LAmB liposomal amphotericin B. Censored cases stopped intermittent treatment before nephrotoxicity occurred. Nephrotoxicity was defined as a >1.5 times increase of baseline serum creatinine levels resulting in an eGFR of less than 40 ml/min/1.73 m^2 during treatment or as electrolyte disorders suspected to be the result of renal damage and requiring cessation of treatment with LAmB at the discretion of the treating clinician.

Outcome	N=18
Median number of days of follow-up, median (range)	741 (145-2543)
All-cause mortality at end of follow-up, number of pts (%)	7 (39)
100 day mortality after start of OPAT, number of pts (%)	0 (0)
1 year mortality after start of OPAT number of pts (%)	1 (6)
Resolution of infection‡, number of pts (%)	13 (72)

Table 2: Outcomes
Legend: ‡ Resolution of infection defined as clinically observed absence of complaints that are likely to be caused by Invasive Fungal Infection in combination with clinically irrelevant or absent abnormalities concordant with Invasive Fungal Infection on High-resolution CT-scan.

DISCUSSION

This study shows that the use of OPAT with LAmB in a 2 or 3 times weekly dosing scheme results in high rates of therapy response in a selected patient population and after confirmation of an initial response to daily IV therapy with LAmB. However, safety issues did arise, resulting in mostly reversible nephrotoxicity and in some cases infection or therapy-related readmission to the hospital.

The majority of patients in this study needed prolonged use of LAmB for the treatment of triazole resistant *A. fumigatus* infections. After the first reports of voriconazole resistant A. fumigatus appeared in 2009 from the Netherlands (30), triazole resistance has now extensively been reported in many regions all over the world (7, 11). Although the prevalence is low in some regions, the rates have been steadily increasing in others (7, 31). The high rates of triazole resistance also impact decision making in patients for whom susceptibility testing is not possible. In many cases, the clinician may fear presence of resistance in case of worsening of clinical or diagnostic parameters after treatment with a triazole even with negative or absent resistance tests. Because of difficulty in establishing triazole-resistance or sensitivity, the clinical suspicion of resistance is becoming an important reason to abstain from further treatment with triazoles and opting for LAmB instead. Fortunately, more possibilities to detect resistance have become available. The impact of resistance testing of invasive aspergillosis using PCR is expected to more effectively guide the clinician in the optimal choice of therapy (32) and is being evaluated in a prospective multicentre study in the Netherlands and Belgium (NCT03121235).

Renal toxicity

Since the introduction of (conventional) amphotericin B as treatment of fungal infections, nephrotoxicity has been a major concern. Nevertheless, nephrotoxicity has significantly decreased after the introduction of the liposomal formulation of amphotericin B (33-36). In particular, patients that need prolonged therapy and are exposed to

high doses over a prolonged period of time are vulnerable for the development of renal adverse events. A decrease in dosage could also be beneficial in mitigating the drug-related renal toxicity. However, nephrotoxicity occurring at the end of the anticipated therapy period has been a reason to stop antifungal treatment prematurely and instead evaluate the natural course of the disease. Importantly, the associated nephrotoxicity was reversible in the majority of cases after cessation of therapy or dose alteration. The occurrence and time course of nephrotoxicity did differ from literature describing patients with daily dosing (37-39). Additionally, some experience in the assessment of the safety of the use of LAmB in an outpatient setting is previously described by Malani et al in 2005 (27). The authors of this study also found high rates of nephrotoxicity; the results are nonetheless not directly comparable due to their inclusion of application of non-lipid formulations of amphotericin B. The mentioned literature reports generally lower rates of reversibility of nephrotoxicity and shorter duration until occurrence of nephrotoxicity. However, a recent study also reports a high rate of reversibility of nephrotoxicity after use of LAmB (40). Possibly, our data supports the theory that nephrotoxicity occurs later and has a higher probability to be reversible when applying LAmB in an intermittent dosing schedule.

Application of OPAT strategies are slowly expanding within the field of infectious diseases and are being implemented in regular practice. Similar to LAmB, intravenous vancomycin therapy is also associated with renal toxicity but has nonetheless been successfully implemented in an OPAT programme for many years now (16, 17). Despite early reluctance, the expected logistic and toxicity-related disadvantages (41, 42) are outweighed by the advantages of a decrease in hospital stay with similar therapeutic effectiveness thanks to the implementation of monitoring of toxicity and therapeutic drug monitoring (15, 17, 43).

Study strengths and limitations.

Despite a nation-wide inquiry, only a small subset of adult patients treated for IFI have been identified. The means by which these patients have been selected to undergo OPAT is inherently biased, i.e. the decision of the clinician to apply this therapeutic strategy has been dependent on many factors, both known and unknown. Since no guideline refers to or advises OPAT with LAmB, and due to lack of supportive literature, physicians may only have elected this approach in specific situations. Additionally, lack of existing intra- or extramural infrastructure to apply OPAT could be a limiting factor. Due to this selection, presumably patients with a relatively favourable prognosis with regard to the IFI were included in our study. Also, the heterogeneity of both the patient population and the different dosings that have been used make it difficult to draw any hard conclusions about efficacy and tolerability. As it is impossible to adjust for all of these factors, the results of our study cannot be directly compared with other cohorts

of patients with IFI. However, the baseline variables that have been presented, summarize the most important characteristics, possibly contributing to identifying potentially eligible patients for this treatment strategy. Only patients with an initial response to therapy with LAmB showing no or only mild prior adverse events related to LAmB use were subjected to this strategy. Hence, the involved physicians balanced the risks of inadequate treatment of invasive fungal disease against the advantages of treatment in the outpatient setting. For future adaptation of this strategy, it is important for the clinician to weigh these factors before deciding on applying OPAT with LAmB.

SUMMARY AND CONCLUSIONS

After documentation of an initial treatment response and in a selected patient group, intermittent therapy with LAmB in the outpatient setting appeared to be a valuable treatment option for IFI. Frequent monitoring of renal function and potassium levels, for example once every week, is strongly recommended for early recognition of nephrotoxicity, as it can also occur during prolonged OPAT. This treatment strategy is expected to provide advantages in costs, decrease of hospital-associated infections and patient's quality of life. Further research will be necessary to expand upon the possibilities that this treatment strategy offers. The identification of eligible patient populations that would most benefit from this strategy as well as further study of the toxicity concerns in this setting, are warrented.

Contributions

RP, AS and RD performed the data collection. RP wrote the first draft of the manuscript. RP, JW and MB were involved in the concept and design of the study. MB, BR, RB and IS acted as local main investigators in their respective centers and provided the data. Analyses were performed by RP in collaboration with MB. All authors critically revised all drafts of the manuscripts and approved the final version.

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Chapter 6

Management of cerebral azole-resistant *A. fumigatus* infection. A role for intraventricular liposomal-amphotericin B?

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Plus est en vous. (Heren van Gruuthuse)

ABSTRACT

Objectives

In the pre-azole era, central nervous system (CNS) infections with *Aspergillus* had a dismal outcome. Survival improved with voriconazole but CNS infections caused by azole-resistant *A. fumigatus* is precluding its use. Intravenous liposomal-amphotericin B (L-AmB) is the preferred treatment option for azole-resistant CNS infections but has suboptimal brain concentrations.

Methods

We describe three patients with biopsy proven CNS aspergillosis where intraventricular L-AmB is added to systemic therapy. 2 patients with azole-resistant and 1 patient with azole-susceptible CNS aspergillosis were treated with intraventricular L-AmB at a dose of 1mg weekly.

Results

We describe 3 patients successfully treated with a combination of intravenous and intraventricular L-AmB. All three patients survived but one patient has serious headache, most likely not related to this treatment.

Conclusions

Intraventricular L-AmB may have a role in the treatment of therapy-refractory CNS aspergillosis when added to systemic therapy.

INTRODUCTION

Few cases of central nervous system (CNS) aspergillosis caused by azole-resistant *Aspergillus fumigatus (ARAF)* have been reported, and almost always with a fatal outcome [1]. Most patients were treated with combination antifungal therapy. Cerebral infections caused by ARAF have almost always a dismal prognosis. Unfortunately, there a no antifungals available that have activity against ARAF and adequately penetrate the brain. Therefore, we added intraventricular liposomal-amphotericin B (L-AmB) to systemic therapy in 3 patients. In this paper, we describe these patients and our clinical experience. All patients provided informed consent.

CASE PRESENTATION

Case 1

An 18-year-old woman with common acute lymphatic leukaemia (ALL) receiving remission induction chemotherapy was diagnosed with a probable invasive pulmonary aspergillosis (IPA). Combination therapy with intravenous (IV) voriconazole and L-AmB (3mg/kg QD) was started. Serum galactomannan was positive (Optical Density (OD) 2.8) and sputum grew an ARAF with the *CYP51A* TR_{34}/L98H mutation. Eight days on therapy, a paresis of the right arm and leg and a right facial nerve paralysis were observed. Brain MRI showed multiple lesions (figure 1A) and a brain biopsy demonstrated hyphae compatible with *Aspergillus* (figure 1C). L-AmB dose was increased to 10 mg/kg and voriconazole (8mg/kg BID) was replaced by posaconazole and dosed at 300mg BID to achieve serum trough concentrations >3mg/L with the hope of achieving therapeutic brain tissue levels. Posaconazole trough concentrations of 5.2 and 6.0 mg/L were documented. Follow-up MRI 15 days after the initiation of therapy showed increased perilesional oedema. During the following 5 months the patient was treated with oral posaconazole 300 mg BID with IV L-AmB daily at 5mg/kg. Six months after diagnosis posaconazole was stopped as cerebral lesions and perilesional oedema had decreased and the arm and leg paresis and facial nerve palsy had improved. Chemotherapy was reinitiated and another 3 months later L-AmB was discontinued. At that time the lesions on brain MRI had decreased in size but not disappeared completely. Unfortunately, six months later the patient was admitted for an epileptic seizure. MRI showed increase in size and oedema around 1 of the 7 lesions. Combination treatment with L-AmB IV (5mg/kg QD) and posaconazole (300mg BID) was reinitiated and was now combined with intraventricular weekly administration of L-AmB (1mg/week). The patient could be discharged with outpatient therapy with L-AmB IV, posaconazole orally and once weekly intraventricular L-AmB using an Ommaya reservoir that was placed for this purpose. The

intraventricular L-AmB therapy was well-tolerated and continued for 4 months. The MRI remained essentially unchanged in these 4 months at that time, the patient was able to walk and cycle independently but has unilateral hand motor dysfunction as the only sequela.

Case 2

A 13-year-old patient with iron overload due to multiple transfusions for beta-thalassaemia was diagnosed with a probable invasive pulmonary and multifocal cerebral aspergillosis 8 months after allogeneic stem cell transplantation (figure 1B). An ARAF was cultured from BAL fluid. Galactomannan in CSF was positive (OD 1.3). Voriconazole (8mg/kg BID IV) and L-AmB (6 mg/kg) were started. Ten days later an epileptic seizure occurred. MRI showed increasing size of the brain lesions and again an ARAF grew from a brain biopsy (table 1). An Ommaya reservoir was placed for the intraventricular administration of L-AmB as well as caspofungin (for details on dosing see table 1). Also intravenous caspofungin (70mg QD) and flucytosine 25mg/kg QID mg) were initiated. With this intervention, the patient improved and lesions decreased in size. Weekly intraventricular administration of L-AmB was continued for 10 weeks and intraventricular caspofungin for 6 months. Systemic therapy with flucytosine, L-AmB and caspofungin was discontinued after 2, 6 and 6 months respectively. No further improvement of the remaining brain lesions was observed after 6 months.

Several years later the patient developed disabling headache. On imaging the skull and dura mater diameter had thickened significantly. A dura biopsy did not lead to a conclusive diagnosis. At last follow-up 9 years post-allogeneic transplant the complains of severe headaches had disappeared but spasticity, occasional epileptic seizures and frontal lobe syndrome have led to severe disability.

Case 3

A 15-year-old girl with common ALL developed aphasia 23 days after chemotherapy initiation. MRI showed 1 lesion in the left frontal and 1 in the temporal lobe. A chest CT showed nodular lesions. BAL sampling was performed. Galactomannan (OD 4.5) was positive and voriconazole-susceptible *A. fumigatus* was cultured (voriconazole MIC 0.5mg/L). Treatment with voriconazole (4mg/kg) and L-AmB (5 mg/kg) was initiated and L-AmB stopped on day 16 when voriconazole drug levels were therapeutic. Despite voriconazole serum levels between 3 and 12 mg/L, a follow-up MRI 3 weeks into therapy showed that lesions had increased in size. Intravenous L-AmB was reinitiated and weekly intraventricular administration of L-AmB 1 mg was started via a Rickham reservoir while voriconazole was continued as well. Follow-up brain MRI's and lung CT at 1 and 2 months into this therapy showed decreasing size of the brain lesions and no increasing lung lesions. A lung biopsy confirmed an invasive aspergillosis infection. Eventually, without

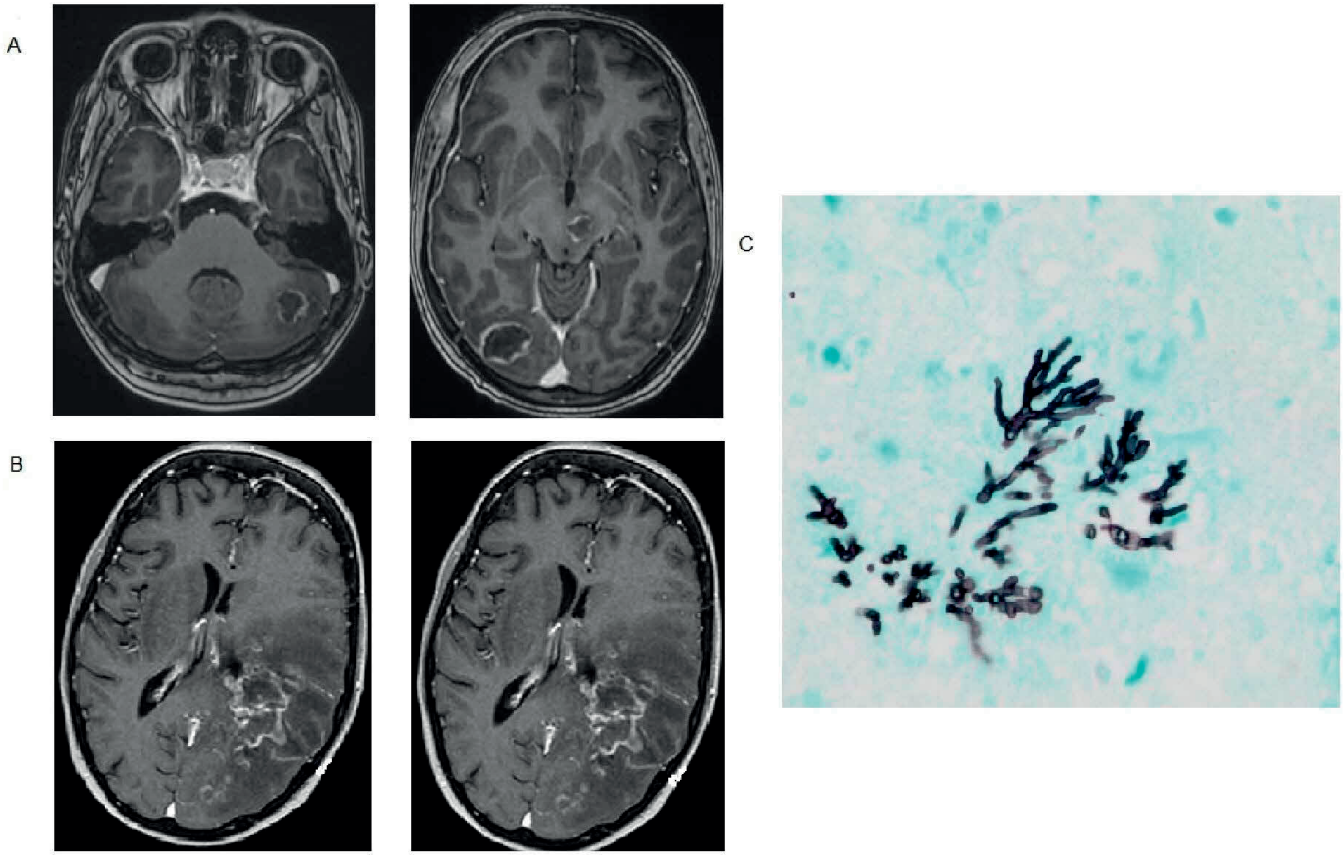

Figure 1: Patient 1, panel A: and patient 2, panel B: MRI showing multiple nodular lesions. Panel C. Brain biopsy shows septated hyphae with dichotomous branching at sharp angles using a gomori methenamine silver stain.

Factors	Patient 1	Patient 2	Patient 3
Sex, Age	F, 18	F, 16	F, 15
Underlying disease	ALL	Thalassemia, allogeneic SCT	ALL
Classification IPA (EORTC/MSG)	Biopsy proven	Biopsy proven	Biopsy proven (brain and Lung)
Culture positive sample	Sputum, brain biopsy	BAL, brain biopsy	BAL
MIC Voriconazole	8	16	0.5
MIC Posaconazole	2	0.5	0.063
MIC Itraconazole	>16	16	0.25
MIC Isavuconazole			0.5
GM value blood	1.3	0.9	0.4
GM value CSF	0.5	1.30	0.5
GM value BAL	2.8	0.36	4.5
Biopsy brain	Positive	Positive	Positive
Treatment regimen (day after diagnosis)	1. Voriconazole + L-AmB IV (3 mg/kg) (d0-d5)	1. L-Amb IV + voriconazole (6 mg/kg) (d0-d10)	1. Voriconazole + L-AmB IV (d0-d10)
	2. Posaconazole + L-AmB IV (10mg/kg) (d5-d26)	2. L-AmB IV + Caspofungin IV (d10-d13)	2. Voriconazole (d11-d20)
	3. Posaconazole + L-AmB IV (3mg/kg) + IT L-AmB* (d26-d191)	3. L-AmB IV + Caspofungin IV/IT$^{\circ}$ (d13-d24)	3. Voriconazole + L-AmB 3mg/kg IV/ weekly IT (d21-d109)
	4. L-AmB 5 mg/kg 3x/ week (d191-d251)	4. L-AmB IV and IT* + Caspofungin IV and IT$^{\circ}$ (d24-d32)	4. Voriconazole (d110-d149)
	5. Treatment re-initiation L-AmB IV + IT *(d386-d515)	5. L-AmB IV and IT* + Caspofungin IV and IT$^{\circ}$ + Flucytosin IV (d32-d97)	5. Voriconazole + L-AmB 3mg/kg IV/ weekly IT* (d149-d176)
		6. L-AmB IV + Caspofungin IV and IT$^{\circ}$ + Flucytosin IV (d97-d201)	6. Isavuconazole + L-AmB 3mg/kg IV and weekly IT (d176-d227)
			7. Isavuconazole + L-AmB weekly IT* (d228-d348)
			8. Isavuconazole (d349-XXX)

Table 1: Clinical and epidemiological characteristics of patients with cerebral azole-resistant invasive aspergillosis

Abbreviations: BAL=bronchoalveolar lavage, CSF=cerebrospinal fluid, F=Female, IPA=invasive pulmonary aspergillosis, EORTC/MSG= European Organization for Research and Treatment of Cancer/ Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group [EORTC/MSG] host factor, F=Female, IPA=invasive pulmonary aspergillosis, IT=intrathecal/intraventricular, IV=intravenous, L-AmB=Liposomal Amphotericin B, MIC=minimal inhibitory concentration. *Intraventricular/intrathecal L-AmB was given at a dose of 1mg weekly $^{\circ}$Caspofungin IT 1 mg QD for 2 weeks1mg 3x/week for 1 month and 3x/week 0.5 mg thereafter

a change in therapy all infections sites improved, as well as the neurological disabilities of the patient. After 3 months of combination therapy, a step-down to voriconazole monotherapy was made. During the following 6 weeks voriconazole levels were suboptimal (range 0.3-2.6mg/L) and unfortunately, 6 weeks later dysarthria developed and an MRI showed that lesions had increased in size. A brain biopsy confirmed the *Aspergillus* infection of the brain. IV and intraventricular L-AmB was reinitiated. IV administration of L-AmB was stopped after two months but intraventricular continued for 8 months while voriconazole was switched to isavuconazole for liver enzyme elevations. Eventually follow-up imaging of lungs and brain showed a good response to therapy. Patient is doing well and has been successfully treated for ALL. During ALL therapy, patient is receiving isavuconazole as secondary antifungal prophylaxis.

DISCUSSION

We describe 2 cases of ARAF and 1 case with azole-susceptible CNS aspergillosis treated with intraventricular L-AmB. Brain infections with *Aspergillus* have a high mortality and survivors are left with at least some neurological deficit [2]. Although voriconazole improved the chances of survival, ARAF now turns back the clock to the amphotericin-B era.

Over the last 10 years, azole-resistance has become an important emerging problem and is associated with a very high mortality [3-6]. When voriconazole resistance is documented in a patient infected with a cerebral *Aspergillus* infection, treatment becomes very difficult. Indeed, few other systemic antifungal agents have been shown to penetrate the brain. Furthermore, the therapeutic effect of new drugs is still unknown [7, 8]. Pharmacokinetic and pharmacodynamic animal data suggest that compared with other formulations of amphotericin-B, L-AmB results in the highest brain tissue concentrations of amphotericin-B and it was effective as therapy in a mouse model of candida encephalitis [9]. Therefore, it is regarded as the preferred second-line therapy for cerebral fungal infections but should, at least initially, be dosed at 5 to 10 mg/kg to achieve therapeutic brain tissue concentrations quickly [1, 10]. For azole-resistant CNS infection, L-AmB can be combined with a second drug but itraconazole, posaconazole and echinocandins do not lead to adequate drug concentrations in CSF or brain tissue with standard dosing regimens [1]. Furthermore, it seems that combination therapy does not lead to synergistic treatment effect *in vitro* against azole-resistant *A. fumigatus* isolates [11]. To improve the CSF and brain penetration higher systemic exposure may be aimed for to subsequently reach higher CSF and brain concentrations that can exert a pharmacological effect even in the setting where pathogen susceptibility is reduced. Furthermore, a damaged blood-brain barrier, which is the case in patients with an angio-invasive *Aspergillus* infection will likely improve the penetration of selected

drugs. With this in mind, we combined posaconazole with L-AmB in the first case [12]. Ultimately, we decided to administer L-AmB directly into the cerebrospinal fluid space as well. The administration was well tolerated with no subjective side effects. Contrary to the first and third case, we observed long-term complications in the second case. We argue that these side effects are probably the result of chronic inflammation and scarring during and after the infection rather than L-AmB or caspofungin mediated toxicity although we cannot exclude with certainty that local combination of drug therapy contributed to these side effects.

Current guidelines do not recommend the use of intraventricular administration of antifungals due to the risk of important adverse events (e.g. chemical meningitis, seizures) [13]. Historically, intrathecal/intraventricular administration of conventional AmB deoxycholate has been and is still being used to treat patients with coccidioidal meningitis. The side effects of intrathecal/intraventricular administration of AmB deoxycholate make it difficult to use and only low doses of typically 0.1 mg are used after which the dose is slowly increased up to 1.0 mg [14]. The reported side effects of AmB deoxycholate, led us to opt for intraventricular L-AmB instead. Based on a theoretical total CSF volume of approximately 100-150 mL in our patients, the administration of 1mg of L-AmB would result in a peak CSF concentration of L-AmB of 10 microgram/mL- which is comparable to the peak plasma concentrations after systemic administration by Groll *et al* [9]. Distribution kinetics as well as clearance mechanism were unknown so we had no knowledge on possible accumulation, hence we started with a presumed safe dose. In case 1 we tried to measure L-AmB in retrospect on left-over CSF fluid but no L-AmB could be detected (limit of detection 0.5 mg/L). In hindsight we argue that the clearance of L-AmB is much more rapid than initially expected. This is explained by the fact that 500 ml of CSF is produced and reabsorbed each day and helps clearing L-AmB. Both the dose and frequency of once weekly intraventricular administration of 1 mg L-AmB might thus be suboptimal and a higher dose as well as a more frequent administration may be preferred for future patients. Intrathecal/intraventricular L-AmB at a higher dose (10mg per administration) for seven consecutive days was shown to be well tolerated in 18 patients with cryptococcal meningitis [15]. Although the exact role of intrathecal/intraventricular L-AmB remains to be defined in patients with an *Aspergillus* infection of the CNS, we propose to initiate intrathecal/intraventricular L-AmB as soon as voriconazole resistance is documented at a dose of 5mg and preferably twice weekly. If available, L-AmB CSF concentration monitoring may guide dosing after the first dose.

Finally, whether local therapy needs to be given in conjunction with systemic therapy is unknown. The benefit of combination therapy may be a more favourable ratio of plasma/brain concentrations and perhaps a longer detainment of adequate CSF/brain concentrations.

Case series like ours have several limitations. In particular, all 3 patients received systemic treatment as well. Therefore, the exact contribution of the intraventricular L-AmB administration cannot be defined. However, it is very unlikely that prospective clinical studies will ever be performed to find the best possible treatment option for very rare infections like CNS aspergillosis. Therefore, treatment should be based on in vitro and animal data and eventually the experience described in case reports and case series can be helpful as well.

In conclusion, 3 patients with a CNS infection with *A. fumigatus* were treated with combination antifungal therapy that included intraventricular L-AmB. All 3 survived but one patient was left with severe sequalae.

Funding

This study was performed as part of our routine work.

Ethical approval

Not required.

Transparency decleration

A.F.A.D.S. has received travel grants from Abvie, Amgen, Roche and Gilead to attend international conferences, not related to this manuscript. B.J.A.R. received research grants from Gilead and MSD outside the context of this study. He also received travel grants from MSD, Gilead, BMS, Jansen-Cilag, ViiV and received personal fees from Gilead, Viiv and Great-Lake pharmaceuticals. He served as an advisor to Gilead, ViiV, BMS, Abbvie, Jansen-Cilag and MSD. RJB has served as a consultant to and has received unrestricted and research grants from Astellas Pharma, Inc., F2G, Gilead Sciences, Merck Sharpe and Dohme Corp., and Pfizer, Inc. All contracts were through Radboudumc and payments were invoiced by Radboudumc. None of the work is related to this manuscript. All other authors: none to declare.

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Chapter 7

Diagnosing invasive pulmonary aspergillosis in hematology patients: a retrospective multicenter evaluation of a novel lateral flow device

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ABSTRACT

Background

Invasive pulmonary aspergillosis (IPA) is a potentially lethal infection in patients with hematological diseases or following allogeneic stem cell transplantation. Early diagnosis is essential as delayed treatment results in increased mortality. Recently, a lateral flow device (LFD) for the diagnosis of IPA was CE-marked and commercialized by OLM Diagnostics.

Methods

We retrospectively analyzed bronchoalveolar lavage fluid (BALf) collected from adult hematology patients from 4 centers in the Netherlands and Belgium. Galactomannan was retested in all samples. All samples were applied to an LFD and read out visually by two independent researchers, blinded to the diagnosis of the patient. All samples were also read out using a digital reader.

Results

We included 11 patients with proven IPA, 68 patients with probable IPA, 44 patients with possible IPA, and 124 patients with no signs of IPA ('controls'). In cases of proven IPA versus controls, sensitivity and specificity were 0.82 and 0.86 (visual readout), and 0.82 and 0.96 (digital readout), respectively. When comparing patients with proven and probable IPA as cases versus controls, sensitivity and specificity were 0.71 and 0.86 respectively. When excluding serum and BALf galactomannan as mycological criteria from the 2008 EORTC/MSG consensus definitions, the LFD was less specific than galactomannan when comparing proven and probable IPA to controls (0.86 vs 0.96, p=0.005), but had similar sensitivity (0.76 vs 0.85, p=0.18).

Conclusions

In this large study of the CE-marked LFD in BALf from hematology patients, the LFD had a good performance for the diagnosis of IPA.

BACKGROUND

Invasive pulmonary aspergillosis (IPA) remains a significant infectious complication in patients with hematological diseases or following allogeneic hematopoietic stem cell transplantation (HSCT)(1). Delayed initiation of *Aspergillus*-specific therapy increases overall mortality, making early diagnosis essential(2). The diagnostic tools currently used in clinical practice consist of fungal culture, direct microscopy (preferably using optical brighteners), and detection of galactomannan (GM), (1-3)-ß-D-glucan (BDG), and/or *Aspergillus* DNA by PCR(3). However, these tools have several limitations in terms of both sensitivity, turnaround time and practicability. The sensitivities of direct tests such as fungal culture or microscopy of samples taken from the site of infection [e.g. bronchoalveolar lavage fluid (BALf)] are as low as 20% to 50%(3). Indirect tests, which detect cell wall antigens produced by *Aspergillus*, such as GM on serum or BALf and BDG in serum, show better sensitivities compared to direct tests(4, 5), but require large numbers of samples to be cost-efficient, as these assays run on 96-well plates. Even when performed in-house, these tests are often run in batches, once or twice weekly, which increases the turnaround time and further delays diagnosis.

More recently, an *Aspergillus*-specific lateral flow device (LFD) has been developed, consisting of a self-contained immuno-chromatographic assay using a mouse monoclonal antibody (JF5) for the detection of an extracellular glycoprotein released by *Aspergillus* during active growth(6) (Figure 1). Because of the single-test design and the minimal sample preparation required, this assay could provide a solution to some of the above-mentioned issues. In addition, preliminary evaluation has shown a sensitivity of 73% and specificity of 90% when applied to BALf(7).

However, except for one recent letter (8), all previously published studies with the LFD have used a prototype device. The current CE-marked LFD (*Asp*LFD, OLM Diagnostics, Newcastle Upon Tyne, United Kingdom) differs in several aspects from this prototype, including the immunoglobulin G subclass of the antibody as well as the chromogen; this could impact the diagnostic characteristics of this test. A small retrospective comparative study (including 9 BALf samples) between the prototype device and the currently available assay showed only fair agreement between both assays (Cohen's kappa 0.43) (9). Although their sensitivities were fairly similar - though lowest in the hematology patients at 68% - the novel assay proved to be more specific(9). Of note, samples were stored frozen between testing with the old and new device, which could partly explain this difference.

The aim of this study was to assess the performance of the recently CE-approved LFD in a large multicenter cohort of hematology patients wo underwent diagnostic bronchoscopy with BALf sampling.

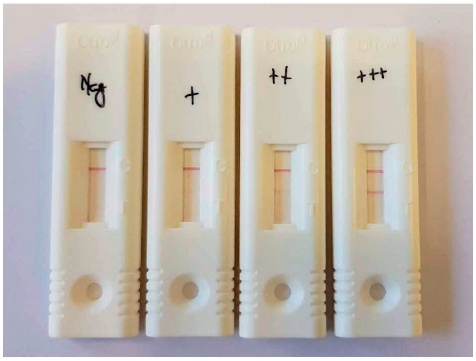

Figure 1: Lateral flow devices, showing from left to right a negative result, followed by increasing test line intensity. The control line is visible at the top, while the test line appears below the control line.

MATERIALS AND METHODS

This retrospective study comprised 247 BALf samples from 247 hemato-oncology patients from 2 academic centers in the Netherlands (Erasmus University Medical Center, Rotterdam, and Radboud University Medical Center, Nijmegen) and 2 centers in Belgium (University Hospitals Leuven, Leuven, and AZ St Jan Bruges, Bruges), collected between 2010 and 2018. Informed consent was waived due to the retrospective nature of this study on stored BALf samples previously collected as part of routine clinical care. The study had no impact on patient management. The LFD was provided by OLM Diagnostics. OLM Diagnostics had no role in the design of this study, its execution, analysis, interpretation of the data, or decision to publish.

Patient selection criteria included (i) age ≥ 18 years; (ii) having an underlying hematological disease or following HSCT; (iii) at least 500 microliter of BALf sample available for analysis stored at ≤ -20°C; (iv) a chest CT scan performed within 7 days of BALf sampling, and (v) access to the full clinical data set. All four participating centers had an integrated care pathway for immunocompromised patients in place, using a standardized protocol: after 72-120 hours of persistent fever unresponsive to broad-spectrum antibiotics, a computed tomography (CT) scan of the chest was performed. Abnormal CT findings were followed by a bronchoscopy and the collection of BALf for extensive microbiologic (including GM detection) and microscopic analysis. Mold-active antifungal prophylaxis was given per institutional policy. We targeted a case:control ratio of 1:2 to reflect the estimated 30% incidence of IPA in hematology patients referred for bronchoscopy in our centers. The following clinical data were collected: demographic data, underlying disease, host factors, serum BDG (±3 days before or after BALf sampling; if available), GM in BALf and serum (±3 days before or after BALf sampling) as determined by the local laboratory, fungal culture results, other microbiological findings, microscopy (with the use of optical brighteners), histopathology (including autopsy)

results, use of mold-active antifungals >24h before bronchoscopy, use of mold-active prophylaxis, absolute neutrophil count, and chest CT scan and bronchoscopy findings. Survival through 12-weeks after initiation of *Aspergillus*-specific therapy was recorded, as well as time to last follow-up.

Case definitions

Patients were classified independently by two physicians as having proven IPA, probable IPA or possible invasive fungal disease in accordance with the revised European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group (EORTC)/Mycoses Study Group of the National Institute of Allergy and Infectious Diseases (MSG) consensus definitions(10). A primary analysis only considered patients with proven IPA as true cases. A secondary analysis also included patients with probable IPA as true cases. Patients considered as not having IPA ('controls') were patients not fulfilling any of the EORTC/MSG clinical and mycological criteria, patients with features suggestive of IPA on pulmonary imaging but with BALf GM optical density index (ODI) <1.0 (see below) and a documented alternative diagnosis (e.g. bacterial) not receiving mold-active therapy, and patients not receiving any specific anti-mold therapy at all who survived for more than 6 months after bronchoscopy.

Study procedures

Repeated GM and LFD testing were performed on all 247 BALf samples at the Belgian National Reference Centre for Mycosis in accordance with the manufacturer's instructions.

Galactomannan enzyme immunoassay: BALf GM detection was performed using the Platelia *Aspergillus* enzyme immunoassay (Bio-Rad, Marnes-la-Coquette, France). All samples were retested in parallel with the LFD to correct for the long-term storage at ≤ -20°C. The GM ODI measured at the local lab was used for classification; the GM ODI tested at the central lab after storage was used for all other analyses. Sample pretreatment and addition of conjugate was performed manually, while incubation, washing, addition of chromogen and stopping solutions, and readout was performed automatically by a BEP-III analyzer (Siemens Healthcare, Erlangen, Germany). Although there is no universally agreed upon threshold for BALf GM positivity, we defined an ODI of ≥1.0 as positive, in line with a recent meta-analysis(11) and with the most recent EORTC/MSG consensus recommendations(12).

***Aspergillus* lateral-flow device:** Briefly, BALf samples were defrosted at room temperature and vortexed. Seventy microliters of BALf were added to the release port on the LFD (*Asp*LFD, OLM Diagnostics, Newcastle Upon Tyne, United Kingdom) and incubated at room temperature for 15 minutes. Hemorrhagic samples or viscous samples due to large amounts of mucus underwent pretreatment in accordance with the manufacturer's instructions, consisting of heating at 100°C for 3 minutes after

adding 300 microliter of EDTA-containing buffer to 150 microliter of BALf, followed by centrifugation at 14,000 g for 5 minutes. Seventy microliters of the supernatant were then applied to the LFD. The LFD was removed from its protective package immediately before applying the sample. Appearance of the control line in the result window showed that the test had run correctly. The appearance of the *Aspergillus*-specific test line was determined after exactly 15 min, with results being recorded as positive if the test line was present. In the absence of a test line, the result was recorded as negative. Each LFD was independently assessed by two evaluators who were otherwise blinded to the final diagnosis (TM and EG). Immediately after reading, the readouts were compared between the 2 evaluators and discordant results were resolved by consensus. During the set-up of this experiment, we noticed a delayed appearance of a test line after more than 15 minutes in some samples that were negative at the 15-minute mark. We therefore performed a second visual readout of all 247 samples between 30 minutes and 1 hour after applying the sample to the LFD. In addition, we investigated the added value of an objective readout method and quantification of results using a digital LFD reader (aLF Reader, QIAGEN Lake Constance, Stockach, Germany). Peak positions were determined using negative and positive controls included in the LFD kit.

Statistical analysis

To calculate sensitivity and specificity with a maximum 95% confidence interval (CI) of 10% width at 80% power, we relied on data previously published by Heldt et al(7), and calculated appropriate sample sizes using the method described by Buderer et al(13). Based on a pooled sensitivity of 73%, a pooled specificity of 90%, and an expected prevalence of 30% in hematology patients undergoing diagnostic bronchoscopy, we estimated a required total of at least 228 patients.

A 2-sided *p*-value of ≤0.05 was considered statistically significant. The diagnostic characteristics of the LFD were compared to those of GM using McNemar's chi-squared, since GM and the LFD are paired observations. Cox regression was used to determine the relation between the LFD result and outcome, controlling for age, gender, HSCT, neutropenia, and prednisone-equivalent dose of ≥ 0.3 mg/kg/day for >21 days.

True positives were defined as patients with proven IPA according to the EORTC/MSG definitions, and true negatives as patients without any evidence of IPA. These strict criteria were used because probable and (to an even greater extent) possible categories (as defined by consensus) are not definitive diagnoses but an assessment of the likelihood of having invasive fungal disease. However, for comparison with previously published findings, our secondary analysis also considered EORTC/MSG defined probable cases as true positives. The EORTC/MSG definitions were used both with and without BALf and serum GM test results included. Indeed, as the GM assay itself is an accepted microbiologic criterion in these definitions, a comparison of the diagnostic performance

of the LFD and GM without the removal of GM from the definition leads to incorporation bias. The negative predictive value (NPV), positive predictive value (PPV), sensitivity, and specificity with likelihood ratios (LRs) and their 95% CI's were calculated.

Cohen's kappa coefficient (with 95% CI) was calculated to measure the agreement between the LFD results (consensus of visual or digital readout) and GM ODI results, between the visual readings of the 2 evaluators, and between visual and digital readout. According to the classification by Landis and Koch, kappa values of >0.8 represent an almost perfect agreement. The effect of long-term storage on GM ODI values was evaluated using the paired Mann–Whitney U test.

Statistical analysis was performed using R v3.5.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Key characteristics of the 247 patients included in this study are shown in Table 1. Eleven patients had proven IPA, 68 had probable IPA, 44 had possible invasive fungal disease, and 124 patients had no IPA, as defined by the EORTC/MSG definitions. *Aspergillus* species were cultured from 30 (12.1%) BALfs, and 75 (30.4%) had a GM ODI ≥ 1.0. Empiric antifungal therapy was started in 21.5% of cases of proven and probable IPA. Retesting of BALf GM at the reference lab was not significantly different from the originally reported value (median GM ODI at time of sampling 0.20 [interquartile range 0.10 - 1.65], versus 0.20 after thawing [interquartile range 0.10 - 1.45], p=0.37). Contingency tables for all subgroups are provided in Supplement 1.

Proven IPA versus controls

The diagnostic performance of BALf GM and the LFD for 11 proven IPA cases versus 124 controls is shown in Table 2. Youden's index was used to determine the optimal optical intensity (OI) cutoff to discriminate between cases of proven IPA and controls. The diagnostic performance of digital readout in this subgroup, using an OI cutoff of 33.15mV (resulting in an area under the curve [AUC] of 0.921), is shown in Table 2. There was excellent agreement between the independent visual readouts of the 2 evaluators (disagreement on 6% of samples, Cohen's kappa 0.86, 95% CI 0.79 – 0.93), and substantial agreement between visual and digital readout (Cohen's kappa 0.65, 95% CI 0.48 – 0.83), resulting in a significantly improved positive predictive value due to a lower number of false positives using digital readout. Sensitivity of visual readout of the LFD was identical to GM (ODI cutoff ≥ 1.0) in this small subgroup of proven IPA (0.82 vs 0.82, p=1.00), but specificity was lower (0.86 vs 0.96, p=0.005). Diagnostic performance of digital readout was identical to that of GM (ODI cutoff ≥ 1.0) in this subgroup.

n	247
Center (%)	
Belgium 1	40 (16.2)
Belgium 2	134 (54.3)
The Netherlands 1	33 (13.4)
The Netherlands 2	40 (16.2)
Age, years (median [IQR])	63 [52, 71]
Male gender (%)	148 (59.9)
Mould-active prophylaxis (%)	17 (6.9)
Disease (%)	
Acute myeloid leukemia	75 (30.4)
Allogeneic SCT	68 (27.5)
Lymphoma	58 (23.5)
Multiple myeloma	14 (5.7)
Acute lymphatic leukemia	10 (4.0)
Myelodysplastic syndrome	8 (3.2)
Autologous SCT	7 (2.8)
Other	7 (2.8)
Neutropenia (%)	118 (47.8)
Use of high-dose corticoids (%)	85 (34.4)
T-cell suppression (%)	125 (50.6)
Severe inborn immune deficit (%)	1 (0.4)
Serum GM ODI (median [IQR])	0.10 [0.07, 0.20]
Serum GM not performed (n [%])	34 (13.8)
***Aspergillus* species (%)**	
A. fumigatus	25 (10.1)
A. flavus	3 (1.2)
A. fumigatus + *A. terreus*	1 (0.4)
A. versicolor	1 (0.4)
Negative	217 (87.9)
Serum ß-D-glucan, pg/mL (median [IQR])	0.00 [0.00, 123.61]
Absolute neutrophil count, /mm³ (median [IQR])	140.00 [0.00, 3200.00]

Table 1: Patient characteristics. SCT = stem cell transplantation, IQR = interquartile range, GM ODI = galactomannan optical density index

Proven or probable IPA versus controls

To allow for a comparison with previous reports on the prototype version of the LFD(14-16) and with other diagnostic tests for IPA, we assessed the diagnostic performance in patients with EORTC/MSG defined proven and probable IPA taken together as cases (n=79) versus controls (n=124), using different cutoffs (≥ 1.0 or ≥ 0.5) for BALf GM positivity (Table 3). Youden's index was calculated again for each BALf GM cutoff. The sensitivity and specificity of visual readout of the LFD were significantly lower than

	LFD (visual readout)	LFD (digital readout)	GM ODI ≥ 1.0	GM ODI ≥ 0.5
Sensitivity (95% CI)	0.82 (0.48, 0.98)	0.82 (0.48, 0.98)	0.82 (0.48, 0.98)	0.82 (0.48, 0.98)
Specificity (95% CI)	0.86 (0.79, 0.92)	0.96 (0.91, 0.99)	0.96 (0.91, 0.99)	0.93 (0.87, 0.97)
Positive predictive value (95% CI)	0.35 (0.17, 0.56)	0.64 (0.35, 0.87)	0.64 (0.35, 0.87)	0.50 (0.26, 0.74)
Negative predictive value (95% CI)	0.98 (0.94, 1.00)	0.98 (0.94, 1.00)	0.98 (0.94, 1.00)	0.98 (0.94, 1.00)
Positive likelihood ratio (95% CI)	5.97 (3.54, 10.06)	20.29 (8.23, 50.04)	20.29 (8.23, 50.04)	11.27 (5.66, 22.43)
Negative likelihood ratio (95% CI)	0.21 (0.06, 0.74)	0.19 (0.05, 0.66)	0.19 (0.05, 0.66)	0.20 (0.06, 0.69)

Table 2: Diagnostic performance in cases of proven invasive pulmonary aspergillosis versus controls. CI = confidence interval. GM ODI = galactomannan optical density index. LFD = lateral flow device.

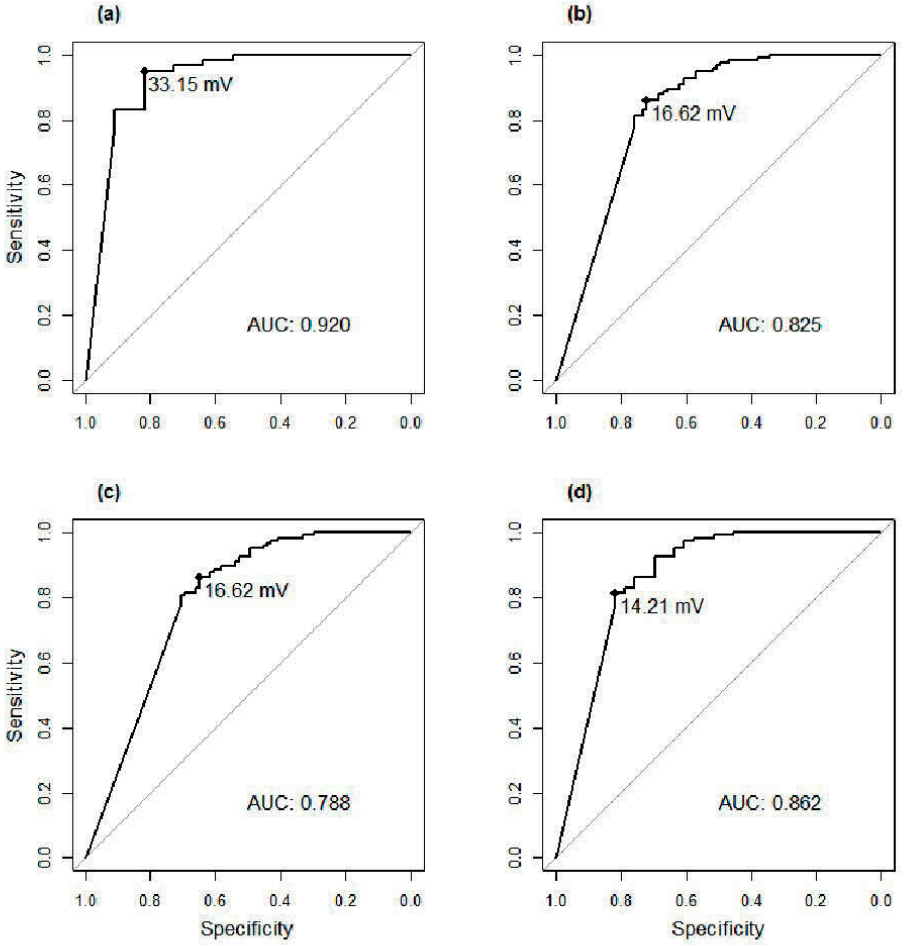

Figure 2: Receiver operating characteristic (ROC) curves of the lateral flow devices in different subgroups of invasive pulmonary aspergillosis (IPA). (a) Proven IPA vs controls. (b) Proven or probable IPA vs controls, galactomannan (GM) positive ≥ 1.0. (c) Proven or probable IPA vs controls, GM positive ≥ 0.5. (d) Proven or probable IPA vs controls, GM excluded as mycological criterion.

Role of BALf GM as mycological criterion	Number of cases	Readout	Cutoff (mV)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	PLR (95% CI)	NLR (95% CI)
BALf GM positive ≥1.0	79	Visual		0.71 (0.60, 0.81)	0.86 (0.79, 0.92)	0.77 (0.65, 0.86)	0.82 (0.75, 0.88)	5.17 (3.25, 8.22)	0.34 (0.24, 0.48)
		Digital	16.62 (AUC 0.826)	0.72 (0.61, 0.82)	0.86 (0.79, 0.92)	0.77 (0.66, 0.86)	0.83 (0.75, 0.89)	5.26 (3.31, 8.36)	0.32 (0.22, 0.46)
BALf GM positive ≥0.5	91	Visual		0.65 (0.54, 0.75)	0.86 (0.79, 0.92)	0.78 (0.67, 0.86)	0.77 (0.69, 0.84)	4.73 (2.97, 7.54)	0.41 (0.31, 0.54)
		Digital	16.62 (AUC 0.789)	0.65 (0.54, 0.75)	0.86 (0.79, 0.92)	0.78 (0.67, 0.86)	0.77 (0.69, 0.84)	4.73 (2.97, 7.54)	0.41 (0.31, 0.54)
BALf GM excluded as mycological criterion	33	Visual		0.76 (0.58, 0.89)	0.86 (0.79, 0.92)	0.60 (0.43, 0.74)	0.93 (0.87, 0.97)	5.53 (3.41, 8.95)	0.28 (0.15, 0.52)
		Digital	14.21 (AUC 0.864)	0.82 (0.65, 0.93)	0.81 (0.73, 0.88)	0.54 (0.39, 0.68)	0.94 (0.88, 0.98)	4.41 (2.95, 6.60)	0.22 (0.11, 0.46)

Table 3: Diagnostic performance in cases of proven/probable invasive pulmonary aspergillosis vs controls.
PPV = Positive predictive value, NPV = negative predictive value, PLR = positive likelihood ration, NLR = negative likelihood ratio, CI = confidence interval, BALf GM = broncho-alveolar lavage galactomannan, AUC = area under the curve.

those of BALf GM (≥ 1.0) in this subgroup (sensitivity 0.71 vs 0.82, p=0.020; specificity 0.86 vs 0.96, p=0.005). Compared to visual readout of the LFD, serum GM had a significantly lower sensitivity (0.37 vs 0.73, p<0.001) and higher specificity (1.00 vs 0.86, p<0.001). BDG was only measured in 9 patients, and could therefore not be compared to the LFD. The receiver operating characteristic (ROC) curves for each subgroup are shown in Figure 2. The agreement between BALf GM and the LFD was substantial, with a Cohen's kappa of 0.61 (95% CI 0.51 – 0.72) for visual readout, and a kappa of 0.63 (95% CI 0.53 – 0.74) for digital readout (cut-off 16.62mV).

Of course, as GM is used as one of the mycological criteria in the EORTC/MSG criteria, this leads to a bias towards GM. Therefore, we omitted BALf and serum GM from the mycological criteria to allow for a direct comparison of the diagnostic characteristics of GM and the LFD. Specificity remained significantly higher for BALf GM (0.86 vs 0.96, p=0.005), with a trend towards a higher sensitivity for BALf GM (0.76 vs 0.85, p=0.18). However, 8 (18.1%) out of the 44 cases of possible invasive fungal disease had a positive LFD by visual readout, all with low OI (median OI of the positive LFDs 19.06mV, interquartile range 14.36mV – 26.11mV).

We found an exponential correlation between the GM ODI and the OI of the LFD as measured by the digital reader (Figure 3). The correlation between both was moderate with an adjusted R^2 of 0.52. Based on the results from this plot, we further identified 2 distinct subgroups, with a breakpoint around a GM ODI of 4.0. Indeed, LFD sensitivity was significantly lower in cases with BALf GM < 4.0 (0.47 vs 0.75, p=0.014) while specificity was similar (0.86 vs 0.88, p=0.909). Furthermore, in cases with a positive fungal culture, the GM ODI was significantly higher (median 6.2 vs 2.75, p=0.046) and there was a trend towards higher OI's of the LFD (median 113.62mV vs 33.87mV, p=0.054). The qualitative result of the LFD was not significantly different in culture positive cases (77.8% positive vs 67.3%, p=0.477). As only 17 patients (6.9%) were receiving mold-active prophylaxis, this subgroup was too small to assess the effect of prophylaxis on diagnostic performance. However, in the subgroup that received empiric antifungal therapy prior to BALf sampling, the sensitivity was significantly lower (0.47 vs 0.77, p=0.32) while specificity was similar (1.00 vs 0.85, p=0.610).

When reading out the LFD between 30 minutes and 1 hour after applying the sample, 7.9% of the initial negative results had become positive, increasing the sensitivity to 0.80 (95% CI 0.69 – 0.88) and decreasing the specificity to 0.79 (95% CI 0.71 – 0.86). In multivariate Cox-regression, the LFD was not a significant predictor of mortality in cases of proven or probable IPA, either when used as a binary variable (p=0.492) or as a continuous variable (p=0.982).

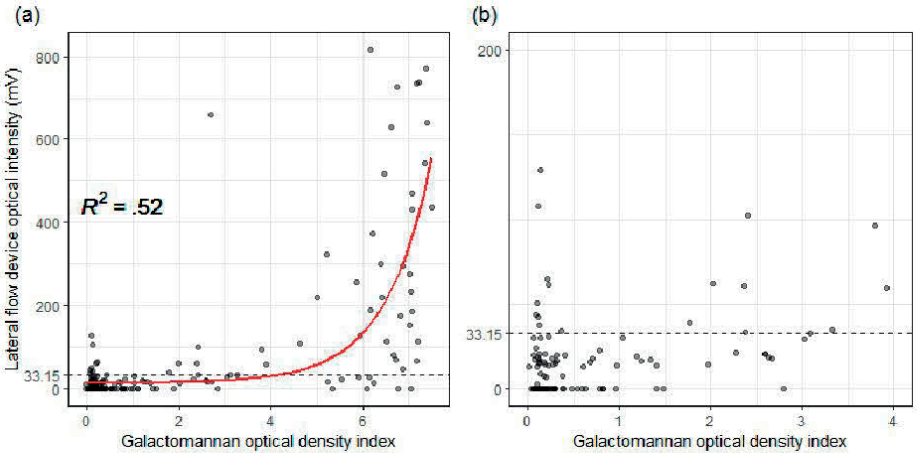

Figure 3: Jitterplot of the galactomannan optical density index vs the intensity of the lateral flow device for all included patients. (a) Overview of all measurements. (b) Zoomed in detail of measurements with galactomannan optical density index ≤ 4.0.

DISCUSSION

We present the largest multicenter trial of a newly CE-approved LFD for the diagnosis of IPA in hematology patients to date, including a total of 247 patients from 4 hospitals in Belgium and the Netherlands, 79 of whom had proven or probable IPA according to consensus definitions. The primary analysis was restricted to the performance of the BALf LFD using only EORTC/MSG proven cases as true positives and cases with no IPA as true negatives. Unfortunately, proven IPA is a rare condition; many patients are thrombocytopenic or in need of supplemental oxygen and are typically not eligible for invasive procedures. In addition, such an analysis introduces disease progression bias, especially when relying on autopsy data. Nevertheless, in this well-documented subgroup, the recently released LFD showed a good diagnostic performance: sensitivity was identical to BALf GM (≥ 1.0), although specificity was significantly lower when read visually. The excellent negative predictive value of 98% in proven IPA could allow clinicians to convincingly withhold mold-active antifungal therapy in at-risk patients with unexplained CT findings. However, generalizing this high NPV to all patient populations should be done cautiously as it is greatly influenced by the prevalence of IPA (Figure 4). Our results are well in line with previously reported studies on the LFD prototype assay(17). Importantly, a digital readout of the LFD greatly increased the performance of the assay in terms of specificity, positive predictive value and positive likelihood ratio, making it identical in performance to GM (Table 2).

Given the rarity of proven cases of IPA, the EORTC/MSG consensus definitions are often used as a diagnostic reference standard. However, these definitions were basically

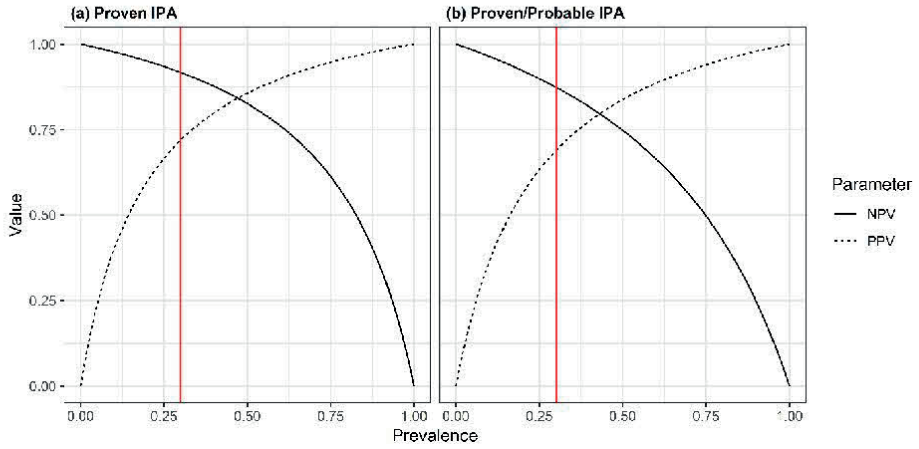

Figure 4: Negative predictive value (NPV) and positive predictive value (PPV) as a function of prevalence of the tested population, in cases of proven invasive pulmonary aspergillosis (IPA) vs controls (a) and in cases of proven and probable IPA vs controls. The red line marks the estimated prevalence used in our study (30%).

developed for clinical and epidemiological research and not for the accurate evaluation of diagnostic tests. Indeed, these criteria are subject to misclassification as well as to incorporation bias (e.g. BALf GM is one of the microbiological criteria for assigning probable disease). Nevertheless, this method of evaluation is still frequently used. We decided to compare the performance of the LFD to the definitions as published and to the definitions with exclusion of GM as a mycological criterion, and found the diagnostic performance of the LFD to be similar to previously published results of the prototype device in hematology patients with proven or probable IPA(7) (sensitivity 0.71 vs 0.67, p=0.744, and specificity 0.86 vs 0.91, p=0.36). This contrasts with the results of a comparative study of 14 cases of proven and probable IPA where samples were tested using both the prototype and CE-marked LFD, which found an increased specificity for the CE-marked LFD(9). Furthermore, we noticed delayed positive reactions with appearance of a test line after 30 to 60 minutes in some cases, which resulted in an increased sensitivity but a decrease in specificity when compared to the consensus reference. A similar effect was seen in a small study of 9 hematology patients with proven or probable IPA(8). This could possibly be explained by non-specific reactions, as the rate of conversion to a positive line is similar in cases and in controls (8.9% and 7.3% respectively). We therefore do not recommend delayed readout.

Interestingly, our study found a significantly lower sensitivity of the LFD in patients with a GM ODI of < 4.0. We clearly demonstrated an exponential relation between the intensity of the LFD test line and the GM ODI. Visual readout of the LFD was reliable with good inter-evaluator agreement, which was confirmed objectively by digital readout.

The quantitative and qualitative results of the LFD on BALf were not predictive of outcome in multivariate Cox regression. This is not unexpected, as similar results were seen with GM testing on BALf(18). This is likely the result of differences in BALf sampling techniques, which are not standardized between physicians and can even differ between procedures by the same physician. This can result in differences in sampling volume, leading to dilution. Furthermore, peripheral lesions and lesions in the upper lobes can be more difficult to reach.

The large sample size of our study allows for an estimation of the performance of the LFD with narrow confidence intervals. Furthermore, the use of independent and blinded observers and the use of a digital reader ensure a high methodological standard for our study. However, this study also has several limitations. The retrospective design implies an artificial prevalence of the disease, thereby influencing the predictive values. We tried to overcome this by selecting cases and controls in a rate similar to what is seen in our centers. However, in settings where IPA is more (or less) frequent, these values will differ. Furthermore, the storage conditions of the samples at ≤ -20°C could theoretically influence the diagnostic performance of the test. We tried to remove this bias by retesting of GM in parallel with the LFD, which did not show any significant degradation over time. However, though similar in chemical structure, it is not guaranteed that the mannoprotein antigen detected by the LFD is equally stable as GM detected by the Platelia enzyme immunoassay.

In conclusion, the CE-marked BALf LFD appears to have a good diagnostic performance for diagnosing IPA in hematology patients, with an even better performance for excluding IPA. The LFD can be used as a point-of-care test, unless the sample is hemorrhagic or heavily contaminated with mucus, in which case a pretreatment in the lab is required. This test could be used as a first-line diagnostic tool in the bronchoscopy suite, given its short turnaround time and economic advantage over GM testing in low-volume settings. However, in view of its low positive predictive value, the LFD is no substitute for additional diagnostic testing (GM, BDG, PCR) to definitively confirm or exclude the diagnosis of IPA.

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Author contributions

TM, JM, KL and AS designed the experiment. TM, AD, AS, and EdK collected the clinical data. TM, AD, EG, BR, PV and MR collected the BALf samples. TM and EG performed the experiments. TM analyzed the data. TM and JM wrote the initial draft. All authors critically revised the initial draft and final manuscript.

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Chapter 8

Detection of azole-susceptible and azole-resistant Aspergillus co-infection by *cyp51A* PCR amplicon melting curve analysis

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What we see depends mainly on what we look for. (John Lubbock)

ABSTRACT

Introduction

The AsperGenius® assay is a multiplex real-time polymerase chain reaction (qPCR) test that allows for simultaneous detection of *Aspergillus species* and identification of the most common mutations in the *A. fumigatus cyp51A* gene conferring resistance (TR_{34}/L98H and TR_{46}/Y121F/T289A) by using melting curve analysis. Mixed infections with azole-resistant and susceptible *A. fumigatus* have rarely been described.

Methods

The AsperGenius® multiplex real-time PCR assay (PathoNostics, Maastricht, the Netherlands) was used on bronchoscopic alveolar lavage (BAL) samples of 91 consecutive patients with a suspected invasive *Aspergillus* infection at the Erasmus MC University Medical Center, Rotterdam.

Results

In 3 cases the AsperGenius® assay indicated the simultaneous presence of wild-type and mutant genes (2 patients with TR_{34}/L98H mutation and 1 patient with TR_{46}/T289A/Y121F mutation) and therefore mixed infections with azole-susceptible and resistant isolates. In one of the three cases, the mixed infection was confirmed by phenotypic antifungal testing of multiple *A. fumigatus* colonies.

Conclusion

The use of a dedicated *A. fumigatus cyp51A* resistance PCR allowed for the detection of mixed infections with azole-resistant and susceptible *Aspergillus* strains. These mixed infections may remain undiagnosed with conventional phenotypic susceptibility testing.

INTRODUCTION

Invasive aspergillosis(IA) is the most frequent pulmonary mould infection in severely immunosuppressed hosts. The introduction of voriconazole has significantly decreased the mortality of IA.[1] However, azole-resistance in *Aspergillus fumigatus* is increasingly reported[2, 3] and its prevalence ranges from 0.6% to 27.8% across studies.[4, 5] The reported mortality of IA caused by azole-resistant strains is very high and varies between 50 and 88%.[2, 6] There are several screening assays for azole-resistance available (VIP™ check, E-test) but fungal broth microdilution susceptibility testing is the standard diagnostic technique. However, cultures often remain negative. Recently, a CE-IVD certified multiplex qPCR was developed(AsperGenius®). It does not only demonstrate the presence of Aspergillus, but also the presence of certain *cyp51A* mutations that confer resistance of *A. fumigatus* to azoles. *Cyp51A* encodes the cytochrome p450 sterol *14a-demethylase*, the target of azoles. There are two main mutation patterns in the *cyp51A* gene that cause azole resistance: TR_{34}/L98H and TR_{46}/Y121F/T289A.[7]

In theory, mixed infections with azole-susceptible and azole-resistant *A. fumigatus* may occur as well but will only be detected if phenotypic testing of multiple colonies is done, a non-standard practice.[8] Here, we describe three cases, in which a co-infection was demonstrated using *cyp51A* molecular analysis on BAL.

METHODS

The methodology of the AsperGenius® assay (PathoNostics, Maastricht, the Netherlands) has been described elsewhere.[6, 9, 10] At Erasmus MC, the AsperGenius® qPCR, a fungal culture (followed by phenotypic resistance testing if positive) and galactomannan(GM) testing is routinely performed on BAL when IA is suspected.

RESULTS

Between December 2014 and February 2017 the AsperGenius assay was performed on BAL samples with a positive GM assay of 91 patients suspected of having IA. In 79%(72/91) of the patients, DNA of *A. fumigatus* or *Aspergillus species* was demonstrated. In 45 of the 72 patients, the resistance PCR was successful and could therefore differentiate between wild-type(WT) and the presence of resistance associated mutations(RAMs). TR34/L98H mutations were detected in eight cases and Y121F/T289A mutations were detected in three cases. Interestingly, in three additional cases, the AsperGenius assay showed the presence of both WT and resistant *A. fumigatus* isolates. So overall, RAMs

were detected in as much as 14 of the 45(31%) patients in which the resistance PCR provided a result.

Case 1

A routine chest CT-scan of a 50-year-old lung transplant recipient showed peribronchovascular consolidations. A BAL was performed, GM was positive (OD 4.0) and *A. fumigatus* was cultured. The only colony that had grown was susceptible to all antifungals tested(EUCAST). Voriconazole was initiated and therapeutic drug levels were documented.[11] Two weeks later he was admitted to the ICU for respiratory insufficiency and antibiotic therapy was initiated. Again, BAL sampling was performed (GM 0.5 OD, fungal culture negative) and a *Parainfluenza virus type 1* PCR was positive. The AsperGenius® PCR on the BAL showed two melting peaks in the supernatant fraction of the BAL. One peak was located at the melting temperature of WT *A. fumigatus* and the other at the melting temperature of the L98H mutated *A. fumigatus*(figure 1a). The patient died 24 days after initiation of voriconazole. The results of the resistance PCR only became available after the patient had died. Retrospectively, the AsperGenius® assay was also performed on residual BAL fluid from the first BAL sample that had been collected. *A. fumigatus* WT-DNA was isolated from both the supernatant and pellet fraction but the resistance PCR also showed a double peak for the L98H probe melting curve analysis(figure 1b). Therefore, the mutated as well as the WT *A. fumigatus* had been present previously but only WT had been detected by conventional phenotypic analysis.

Case 2

A 60-year-old woman received corticosteroids for 5 months for pyoderma gangrenosum and was admitted for respiratory failure. Ceftriaxone and ciprofloxacin were started. The next day mechanical ventilation was needed. Chest CT-scan showed several spherical consolidations. Oseltamivir and antifungal treatment(voriconazole and caspofungin) were added empirically. A BAL showed a GM of 5.6 OD and *A. fumigatus* was cultured. Liver toxicity led to a switch from voriconazole to liposomal-amphotericin-B(L-AMB). The phenotypic resistance test(EUCAST) of the Aspergillus strains cultured from BAL fluid showed that 5 of the 6 *A. fumigatus* colony forming units (cfus) had a minimal inhibitory concentration(MIC) of 0.25mg/L for itraconazole, voriconazole, and posaconazole, while the MIC of the sixth cfu was >8mg/L for itraconazole, 4mg/L for voriconazole and 0.5mg/L for posaconazole. The qPCR of the BAL sample confirmed the presence of *A. fumigatus* and the resistance PCR showed melting curves specific for mutant(T289A/Y121F) and WT-DNA(figure 2a/b) indicating a mixed infection. Patient died of progressive multiple organ failure 10 days after the start of L-AMB.

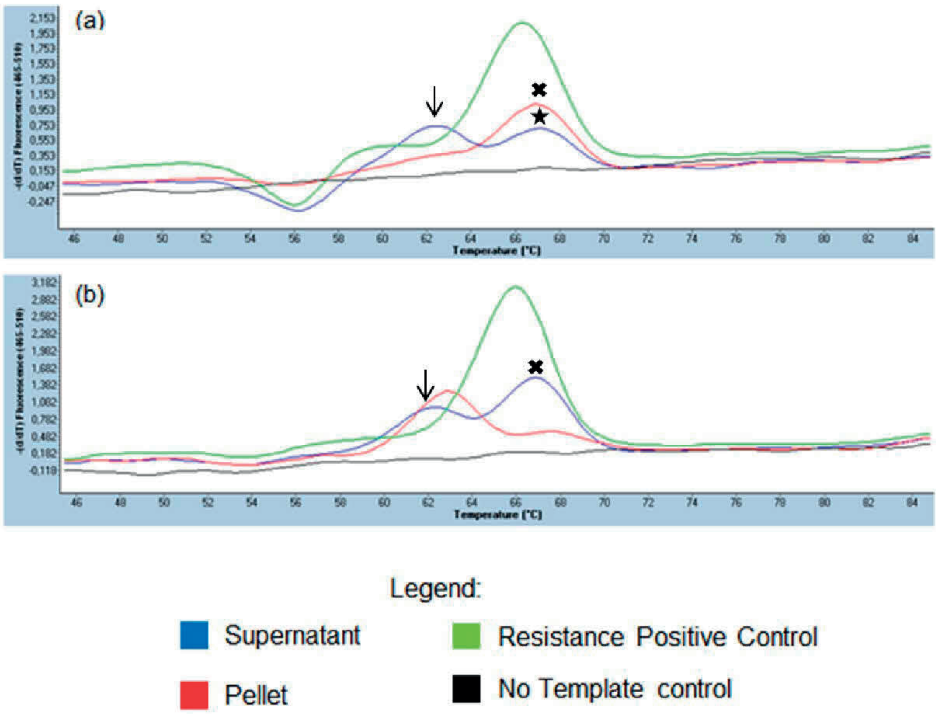

Figure 1: (a) Case 1 melting curves using the L98H mutation probe. Two melting peaks were detected for the supernatant fraction. One peak was specific for wild-type DNA (marked by an arrow), the other peak was specific for L98H mutant DNA (marked by a star). A specific melting peak was detected for the pellet extract (marked by a X), and corresponds to the mutant positive control, indicating L98H mutant DNA. (b) Case 1 melting curves using the L98H mutation probe on leftover BAL: A double peak for wild-type (marked by an arrow) as well as mutant (marked by a X) is present in the supernatant fraction indicating that low concentrations of *A. fumigatus* mutant L98H DNA were present in the supernatant of the BAL.

Case 3

A seven-year-old patient, recently diagnosed with AML, was admitted for dyspnoea. A chest CT-scan showed multiple nodular lesions. A BAL was performed and GM was positive(OD 3.9). Combination therapy was initiated with L-AMB and voriconazole and therapeutic voriconazole drug levels were documented. The BAL culture showed one cfu of *A. fumigatus* susceptible to all antifungals tested(EUCAST). AsperGenius® qPCR confirmed the presence of *A. fumigatus* DNA and the resistance PCR showed both L98H mutant DNA and WT-DNA(figure 3). Voriconazole was discontinued after two weeks and L-AMB was continued for 12 weeks. A new CT-scan, performed a month after L-AmB discontinuation, showed that the lesions had increased in size. Pathology of a CT-directed biopsy of one of the lesions showed only chronic interstitial inflammation and cultures remained negative. Unfortunately, the patient suddenly died 13 months later.

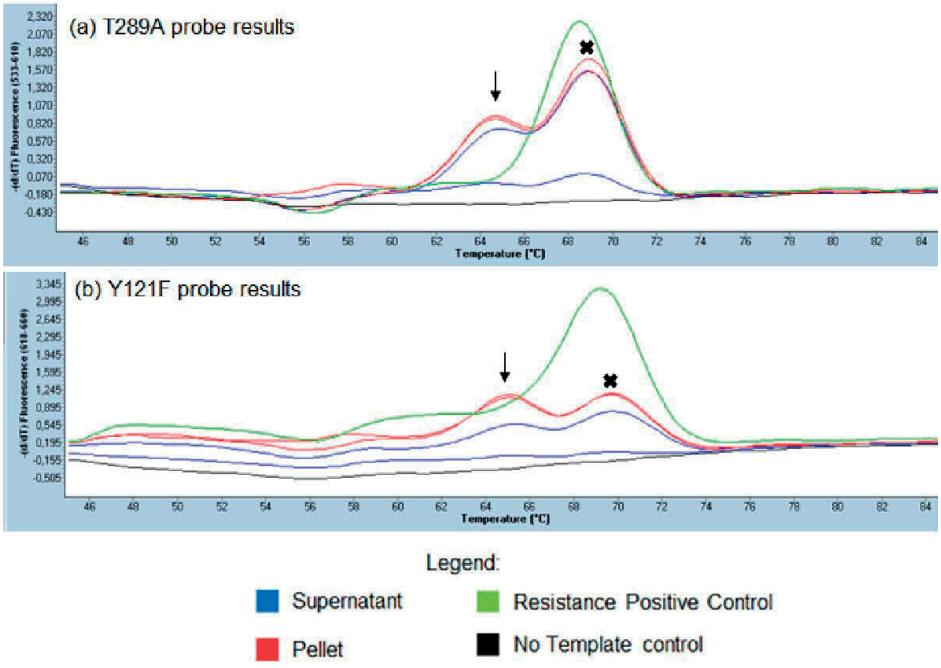

Figure 2: Case 2 mutation analysis. (a) T289A mutation analysis and (b) Y121F mutation analysis: Multiple specific melting peaks were detected for all extracts (pellet and supernatant), which were mutant (marked by a X) and wild-type (marked by an arrow) indicating the presence of wild-type and mutant DNA. The green melting curve is indicating the positive control representing the mutant marker.

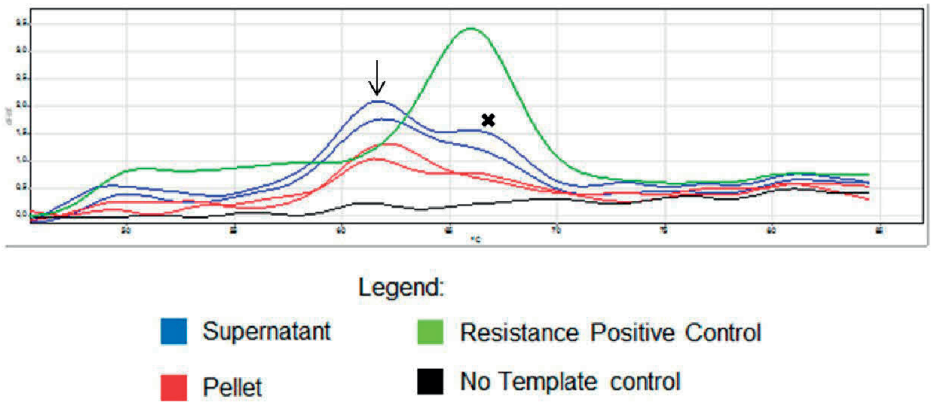

Figure 3: L98H mutation analysis for case 3. Two melting peaks were detected for the supernatant fraction. One peak was specific for wild-type DNA (marked by an arrow), the other peak was specific for L98H mutant DNA (marked by a X).

DISCUSSION

We describe three patients with an IA infection in which WT as well as mutant *cyp51A* DNA from *A. fumigatus* was detected. In one patient, the mixed infection was confirmed by phenotypic resistance testing of multiple *A. fumigatus* colonies. To the best of our knowledge, this is the first report in which co-infection of azole-resistant and susceptible *A. fumigatus* was detected by a molecular assay.

A. fumigatus mixed-infections are rarely recognized. A recent paper described 3 cases of culture confirmed *A. fumigatus* mixed infection of susceptible and resistant isolates.[8] However, the majority of BAL samples from patients with IA are culture negative. As such, the presence of azole resistance may remain undetected.[6, 12, 13] In 2 of our 3 cases BAL cultures showed growth of *A. fumigatus* but phenotypic testing failed to show resistance in 2 of the 3 cases. As growth of only 1 colony of *A. fumigatus* was present in case 1 and 3, only this colony could be tested phenotypically which might explain the discrepancy between the resistance PCR and the culture results. Thus, performing a resistance PCR directly on BAL may yield additional information and may avoid the reporting of very major errors(i.e. sensitive result when resistant *A fumigatus* is present).

Treatment with voriconazole is associated with a high risk of treatment failure and mortality in patients with azole-resistant *A. fumigatus*.[2, 6] Non-culture based methods of resistance testing therefore have the advantage that appropriate antifungal therapy can be initiated immediately and hopefully reduce the risk of treatment failure.[2, 6] *A. fumigatus* is genetically diverse and multiple genotypically different isolates can be obtained from multiple BAL samples of one patient.[14] Recently, a case report described the presence of different *Aspergillus* genotypes in different body compartments.[15] Mixed cultures of *A. fumigatus* strains are present in environmental and clinical samples.[8, 16] One of the isolates can be dominant and can disseminate, causing disease. The presence of different isolates with different susceptibility profiles complicates the diagnosis and management of IA.[8, 14]

These observations show that even if an azole-susceptible *Aspergillus* isolate is cultured, the patient can still harbour an azole-resistant isolate in regions where TR_{34}/L98H and TR_{46}/Y121F/T289A environmental strains are endemic. As described in case 1, two BAL samples were performed. The first BAL sample was performed before and the second BAL two weeks after azole treatment was initiated. The first BAL sample grew *A. fumigatus* susceptible to voriconazole. The second BAL was culture negative but the PCR analysis showed a mixed infection with TR_{34}/L98H mutated and WT *A. fumigatus*. In retrospect, mixed infection could also be demonstrated on the first BAL sample. We therefore suggest that in regions where azole-resistance has been described, at least 5 and preferably all distinct *A. fumigatus* colonies are phenotypically tested for

the presence of azole-resistance and if possible the BAL sample itself is tested for the presence of known *cyp51A* mutations that confer resistance to azoles. Importantly, it should be noted that the AsperGenius® detects only the 2 most common mutations found in azole-resistant and *A. fumigatus* isolates with other mutations or non-*cyp51A* mutations will remain undetected. Therefore, the assay should be used in addition to conventional susceptibility testing. Furthermore, in vitro simulations showed that a ratio of mutant:WT below 1:5 will also remain undetected.

In conclusion, the AsperGenius® assay can detect mixed infections with azole-resistant and azole-susceptible *A. fumigatus* isolates enabling on-time and targeted therapy. Importantly, it can detect mixed infections when conventional fungal cultures are negative.

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Chapter 9

PCR based detection of azole resistance in *A. fumigatus* to improve patient outcome: The azole resistance management study (AzoRMan). A prospective multicentre observational study.

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No author list is provided because this is an unpublished interim analysis of an ongoing study

Peer review proces hangt te veel af van de goodwill van anderen

ABSTRACT

Introduction

Invasive aspergillosis (IA) is the most common mould infection in patients with acute leukaemia or graft-versus-host-disease. A relatively low attributable mortality is observed when IA is diagnosed early and treatment with an azole initiated promptly. However, azole resistance in *Aspergillus fumigatus* is increasingly reported in Europe and is associated with a higher mortality. Unfortunately, phenotypic susceptibility testing of moulds is time-consuming, not widely available and most importantly fungal cultures remain negative in the majority of the patients with IA. AsperGenius® is a multiplex real-time polymerase chain reaction (PCR) assay that allows for a simultaneous detection of *Aspergillus* species and identification of the most common mutations in the *A. fumigatus* CYP51A gene conferring resistance. The use of this PCR has the potential to diagnose azole resistance more frequently and faster and should therefore facilitate the initiation of appropriate therapy at an earlier point in time. A fast diagnosis and correct treatment of azole-resistant aspergillosis should lead to an improved outcome.

Methods

All Dutch academic haematology units agreed on a consensus IA management protocol. In this protocol, the AsperGenius® PCR is used directly on broncho-alveolar lavage fluid (BALf) to accelerate the diagnosis of azole-resistance and change antifungal treatment accordingly if resistance is detected. This management protocol was used in the Azole Resistance Management Study (AzoRMan), a prospective multicentre observational study in immunocompromised adult haematological patients, with pulmonary lesion(s) on chest CT scan that are undergoing a diagnostic bronchoscopy to confirm or rule out IA. The objective of this study is to evaluate the impact of the AsperGenius® PCR on the outcome of patients infected with an azole-resistant *A. fumigatus*. The study also makes prospective monitoring possible of the prevalence of the 2 most common resistance-associated mutations (RAMs) in the *A. fumigatus* CYP51A gene and in particular in culture-negative cases of IA.

Results

As of December 2019, 212 patients have been included from 9 centres. Galactomannan was positive (1.0 or higher) on BALf in 46/190 patients (24%) with available GM result. The AsperGenius® species and fumigatus PCR was positive in 40% and 29% of the patients respectively. In patients with a positive or negative galactomannan on BALf, the *Aspergillus* species PCR was successful in 78% and 28% of patients, respectively. RAMs were documented in 4 patients (8.5%) of a total 47 patients in whom the resistance PCR was successful.

Conclusion

The majority of patients with a haematological disease that undergo BALf sampling to confirm or rule out an IA, do not have this infection. In the 47 patients in whom the resistance PCR was successful, the prevalence of CYP51A gene mutations was 8.5%. Given the fact that in only 47 of the 195 with available AsperGenius® PCR result, the resistance PCR was successful, the sample size of the study population needs to be increased substantially in order to answer the primary research question.

INTRODUCTION

Invasive aspergillosis (IA) is the most common mould infection in immunocompromised haematological patients. A relatively low mortality is observed when diagnosis is made early and treatment with voriconazole or isavuconazole, the first choice of treatment, is initiated promptly (1, 2). However, azole-resistance in *Aspergillus fumigatus* is increasingly reported in Europe (3) and is mostly caused by resistance associated mutations (RAMs) in the *cyp51A* gene, encoding for the target enzyme of azoles 14alpha-methylase. Fungal susceptibility testing is difficult, time consuming and not widely available. Furthermore, cultures remain negative in the majority of patients with IA. AsperGenius®, is a CE certified multiplex real-time polymerase chain reaction (PCR) assay that allows for a simultaneous detection of *Aspergillus species* and identification of the most common mutations in the *A. fumigatus* CYP51A gene conferring resistance to itraconazole, voriconazole and posaconazole (4). The use of this PCR results in faster diagnosis of azole-resistance and thus the initiation of appropriate therapy at an earlier point in time. Furthermore, the advantage of this PCR is that it can detect azole-resistance in culture-positive but also culture-negative broncho-alveolar lavage samples. Recently, it has been shown that azole-resistance is associated with an increase in mortality of 21% compared to azole-susceptible IA cases 42 days after the start of antifungal treatment. Mortality of azole-resistant IA is as high as 62% three months after diagnosis (5). Unfortunately, from a global perspective the highest incidence of IA has been observed in The Netherlands and therefore, strategies to tackle the impact of azole resistance on outcome are urgently needed. After extensive discussions and a face-to-face meeting with representatives of all university medical centres in the Netherlands as described in chapter 2, a consensus diagnostic and therapeutic protocol was agreed upon (6). In this protocol, the AsperGenius® PCR is part of the diagnostic protocol and antifungal treatment is changed if resistance is detected (see figure 1). The value of this protocol will be evaluated in the study described below that we call the Azole Resistance Management (AzoRMan) study. The study has two main objectives. The first objective is to improve the outcome of patients infected with an azole-resistant *A. fumigatus* by the early detection of resistant associated mutations and with this the early initiation of the most appropriate therapy (liposomal-amphotericin B) (7). The second objective is to monitor the prevalence in the Netherlands of invasive aspergillosis due to strains carrying the TR_{34}/L98H or the TR_{46}/T289A/Y121F *CYP51a* resistance associated mutations using PCR in particular in culture-negative cases of IA.

METHODS

Study design

This is a prospective multicentre observational study performed in 9 centres in The Netherlands and 2 centres in Belgium. The study population consists of patients with an underlying haematological disease (AML, allogeneic stem cell transplant etc.) aged 18 years and older. These patients are eligible for the study if they are presenting with a new pulmonary infiltrate on chest CT-scan that may be caused by an invasive fungal infection and are planned to undergo or have just undergone a bronchoscopic alveolar lavage (BAL). The treating physician is planning to start voriconazole or isavuconazole or posaconazole after the BAL has been sampled (or after the galactomannan (GM) and PCR result become available) or has already started voriconazole or isavuconazole or posaconazole before BAL sampling.

AsperGenius® PCR

The Dutch centres send BAL sample of at least 1 ml, preferably 2 ml to Erasmus University Medical Centre where the AsperGenius® PCR is done routinely following the manufacturer's instructions (Pathonostics, Maastricht, The Netherlands) (4, 8, 9). As already mentioned, when performed on BALf the AsperGenius® PCR allows for the rapid detection of *Aspergillus* DNA and the absence or presence of the 2 most prevalent azole resistance-associated mutations (TR_{34}/L98H or the TR_{46}/T289A/Y121F in CYP51a*)*. The use of this PCR will therefore decrease the time to detection of azole-resistance compared with the time consuming phenotypic resistance testing. Furthermore, at least 50% of IA cases are culture-negative and in these patients phenotypic testing is not possible.

Treatment protocol (figure 1)

Haematological patients undergo BAL sampling as per standard of care. The diagnostic and treatment protocol that was implemented in the study centres is described in figure 1. In brief, if azole-resistance is detected with PCR or standard phenotypic susceptibility testing, the treating physician will switch from the triazole to liposomal-amphotericin B 3mg/kg IV. In case of treatment limiting toxicity of liposomal-amphotericin B, the use of an echinocandin in combination with posaconazole is suggested aiming at serum Ctrough levels of 3-4mg/L. The rationale and feasibility of posaconazole high-dose has been described elsewhere (chapter 4). Step-down therapy from liposomal-amphotericin B is allowed to oral therapy with posaconazole after at least 2 weeks of liposomal-amphotericin B therapy and after a documented clinical and/or radiological response. In this step-down strategy, posaconazole serum Ctrough levels of 3-4mg/L are aimed for. Importantly, step-down to posaconazole will not be done if an *A. fumigatus* strain

with a MIC of >0.5 microgram/ml is cultured. As an alternative to posaconazole step-down, IV liposomal-amphotericin B 5mg/kg thrice weekly can be given as well following our experience with Outpatient Parenteral Antifungal Therapy (OPAT) with liposomal-amphotericin B as described in chapter 5.

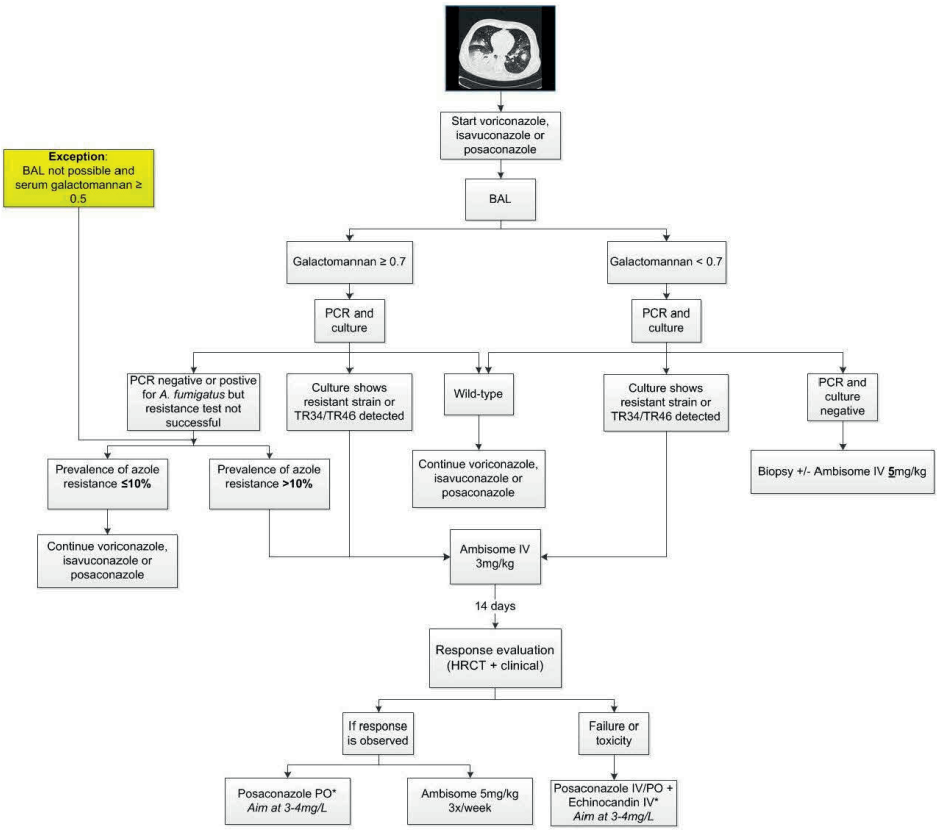

Figure 1: Treatment protocol for Azole Resistance Management (AzoRMan)-study.
MIC=Minimal Inhibitory concentration; IV=Intravenously. *Posaconazole HD can only be considered as treatment option when the MIC (EUCAST) ≤ 1g/dL. HRCT= High Resolution CT scan, PCR=polymerase chain reaction, PO=by mouth, BAL= broncho-alveolar lavage.

Primary and secondary endpoints

The primary endpoint of the study is the proportion of patients with azole-resistance IA that have treatment failure and this will be compared with a fixed 75% treatment failure incidence (as described in the retrospective AsperGenius® PCR validation study (8)). The secondary endpoints of this study are prevalence of azole-resistance documented by fungal culture and resistance PCR and the proportion of patients included in this study that have died 6 weeks after the start of appropriate antifungal therapy.

The latter will be compared with a fixed 50% overall mortality in the same way as the primary endpoint analysis is done.

Sample size calculation

The use of real-time detection of azole-resistance allows for a proactive change in therapy from the first line voriconazole therapy to other agents as soon as resistance is detected. In patients that start a non-azole therapy right from the start, the antifungal therapy can be changed to voriconazole as soon as the absence of RAM is documented. The goal is to demonstrate that this approach reduces the incidence of antifungal treatment failure. Based on previous research, we can assume that treatment failure is seen in 75% if the PCR is not performed and therefore voriconazole is continued in patients with RAMs (8). We assume that treatment failure will be reduced to 35% when the antifungal therapy is changed to liposomal-amphotericin B in the presence of RAMs. Using these percentages, we will need 15 patients with detected RAMs that are switched to non-azole therapy (e.g. liposomal-amphotericin B) or in which liposomal-amphotericin B is continued (if it was given as the primary therapy) to show with a statistical power of 90% that this approach leads to a decrease in treatment failures to a number significantly less than 75%. With an estimated prevalence of CYP51a resistance of 7.5%, this means that 200 patients are needed in which the CYP51a PCR is successfully performed. As we can expect that the AsperGenius® resistance PCR will be successful in 75-80% of the samples that are tested, this means that we need galactomannan positive BAL samples from 280 patients. The study will end when at least 15 patients with CYP51a mutations have been found. Sample size calculation was done with http://powerandsamplesize.com/Calculators/Test-1-Proportion/1-Sample-Equality with 0.75 and 0.35 as parameters and 90% power.

RESULTS

The results presented here are preliminary. Some data are still missing because not all centres have completed the electronic case report form (eCRF) for all the included patients yet.

Baseline characteristics

As of December 2019, 212 patients have been included in the study. The median age of the patients in the study is 64 years and the majority is male (table 1). The most frequent underlying haematological malignancy is acute myeloid leukaemia (60%), followed by myelodysplastic syndrome (17%) and acute lymphoblastic leukaemia (6%). 45 patients had received an allogeneic stem cell transplant.

Baseline characteristics	Results	Data missing
Median age (IQR)	64 (55,69)	20
Male sex (IQR)	86 (67%)	83
BMI (kg/m2) (IQR)	24 (22,28)	84
Allogeneic stem cell transplant recipient	45 (35%)	85
Autologous stem cell recipient	6 (5%)	
Underlying haematological disease	128	84
AML	77 (60%)	
MDS	22 (17%)	
ALL	7 (6%)	
CML	6 (5%)	
NHL	5 (4%)	
Other	11 (9%)	

Table 1: Baseline characteristics.
Abbreviations: IQR: Interquartile range; BMI=Body Mass Index, AML=acute myeloid leukemia; MDS=myelodysplastic syndrome; ALL=acute lymphoblastic leukaemia, CL=chronic myeloid leukemia; NHL=non-Hodgkin lymphoma.

Performance diagnostics

The results of galactomannan (GM) were available for 190 patients. If a GM test result with an optical density ≥1.0 is considered positive, 46 (24%) patients had a positive BAL GM with a median optical density of 5.0 IQR (3.0 and 5.9). In 11 patients (8.5%), a positive culture for *Aspergillus species* was present of a total of the 130 patients with available information on culture results (n=10 *A. Fumigatus, n=1 A. Terreus)*. Results of the AsperGenius® PCR were available for 195 patients in this interim analysis. The AsperGenius® species PCR was positive in 77 of 195 (40%) patients. The *Aspergillus Fumigatus* PCR is only performed when the *species* PCR is positive. A positive *fumigatus* PCR was observed in 57 patients (29%). Of these 57 patients, the resistance PCRs that detected the TR34 and the TR46 mutation pattern were successful in 47 (82%) and 45 (79%) patients, respectively. In 4 patients, a resistance-associated mutation (RAM) was identified (3 TR34 and 1 TR46). Thus, in the patients in whom the resistance PCR was successfully performed, the prevalence of RAMs was 4 of 47 or 8.5% (95% C.I. 0.005-0.165). The AsperGenius® PCR was performed in 138 patients with a negative BAL GM (optical density <1). In this subpopulation, a positive AsperGenius® species and *A. Fumigatus* PCR was documented in 38 (28%) and 26 (19%) patients respectively. The opposite (a positive GM in a patient with a negative AsperGenius® species PCR) was observed in 7 patients only. In patients with a positive GM, 78% (36/43) and 67% (31/43) had a positive *Aspergillus species* and *fumigatus* PCR; respectively. In 3 patients with positive GM, data are missing on the result of the AsperGenius® PCR. The CYP51A resistance PCR was successfully performed in 29 of the 31 patients with a positive A. *fumigatus* PCR. An *Aspergillus Fumigatus* with a RAM was documented in 1 patient with a negative GM on BAL.

	Galactomannan		
	<0.5	0.5-0.9	>1
BAL Galactomanan	129	15	46
***Aspergillus species* positive**	32	6	36
***Aspergillus species* negative**	88	7	7
***A. fumigatus* positive**	20	6	31
***A. fumigatus* negative/NP**	101	7	12
***A. Terreus* positive**	1	0	1
TR34/L98H and TR46/T289A/Y121F WT	10	4	26
TR34/L98H and TR46/T289A/Y121F not successful	6	1	2
TR34/L98H WT and TR46/T289A/Y121F not successful	2	1	0
TR34/L98H not successful and TR46/T289A/Y121F WT	1	0	0
TR34/L98H Resistant and TR46/T289A/Y121F WT	1	0	2
TR34/L98H WT and TR46/T289A/Y121F Resistant	0	0	1

Table 2: Microbiological results of diagnostic tests performed.
Abbreviations: GM=galactomannan, BAL=bronchoscopic alveolar lavage, NP= not performed, WT=Wild-type

Outcome

Not all patients started antifungal therapy. Of those who started therapy and of which we have information on antifungal therapy (n=98), as expected the majority (80%) of the patients started with azole monotherapy: 68 patients with voriconazole, 9 with posaconazole and 1 patient with isavuconazole. 14 patients (14%) started with combination antifungal therapy although this should not have been the case according to the study protocol: 7 patients with voriconazole and anidulafungin, 5 patients with voriconazole and liposomal-amphotericin B and 2 patients with posaconazole combined with anidulafungin. 3 patients started with echinocandin monotherapy: 2 and 1 with anidulafungin and caspofungin, respectively. Centres have been informed again that patients in which combination therapy is (planned to be) initiated should not be included. In 36 patients, antifungal therapy was changed 72 hours or later after start of the first antifungal drug. Antifungal therapy was changed by adding or switching to another antifungal drug. 42 patients (20%) died within 12 weeks after BAL sampling of a total of 101 patients with available data on the 12-week outcome.

Age (years)	Sex	Disease	BAL GM	Cult	MIC per antifungal				Died
					VOR	ITRA	POS	ISA	
66	M	AML	1.6	+	16	1	0.25	16	No
52	F	AlloTx/HL	0.3	+	2	2	0.25	8	No
55	M	AlloTx/FL	4.8	+	8	4	1	8	Yes
48	M	AML	5.6	-					Yes

Table 3: Data on all azole-resistant cases.
Abbreviations: M=Male; F=female; BAL=broncho-alveolar lavage, Cult= Culture; MIC= minimal inhibitory concentration; Vor= Voriconazole; ITRA=itraconazole, POS=posaconazole; ISA=isavuconazole.

DISCUSSION

As shown in chapter 5, azole-resistance is associated with a 25% higher overall mortality three months after the start of antifungal therapy and the initiation of initially inappropriate antifungal therapy is associated with reduced survival (5). Unfortunately, from a global perspective the azole-resistance prevalence is probably the highest in The Netherlands (3, 5). The AsperGenius® PCR is a CE certified and commercially available multiplex real-time PCR that can demonstrate *Aspergillus* DNA and is able to simultaneously detect the presence of the most frequently described *CYP51a* mutations that confer resistance of *A. fumigatus* to itraconazole, voriconazole and posaconazole (4). Obviously, the advantage of this PCR is that it can detect azole-resistance in culture-positive but also culture-negative BAL samples. Therefore, it can help with the detection of azole resistance at an earlier time point in the course of the disease. The clinicians that are treating these patients face a devil's dilemma. Because cultures of most patients with IA remain negative (11), the first hint for the clinician that the *Aspergillus* strain infecting the patient might be azole-resistant will be at the time of clinical failure of azole therapy. However, the mortality of patients in which a switch to another antifungal therapy is made at the time of clinical treatment failure is very high. Therefore, one may consider initiating therapy with an antifungal of another class than the triazoles (e.g. liposomal-amphotericin B or an echinocandin) or with combination therapy right from the start. However, this comes with toxicity and these other antifungals can only be given intravenously. To evaluate if the use of PCR can help the clinician, a meeting was organized with representatives of all Dutch university hospitals and resulted in the AzoRMan-treatment protocol of this study (figure 1). This protocol was subsequently implemented in the academic haematology treatment centres in The Netherlands (chapter 2) (6). During the course of the study other non-academic centres in the Netherlands (Meander MC) and centres in Belgium (AZ Sint-Jan Brugge and University Hospitals Leuven) joined the study. Indeed, azole-resistance proved to be an important emerging problem in Belgium as well (12, 13).

To the best of our knowledge, the AzoRMan-study is the largest prospective study evaluating the value of real-time PCR diagnosis of azole-resistance. This study evaluates if PCR based therapy will help with the timely initiation of the most appropriate antifungal therapy in order to improve the outcome of azole-resistant IA. In this study patients with an underlying haematological malignancy are included when BAL sampling is ordered by the clinician to confirm or exclude the presence of an invasive fungal infection in the patient. Given the fact that the data described above are an interim analysis, the results should be considered preliminary and should be interpreted with this in mind. GM or PCR was positive in 24% and 40% of the patients, respectively. Therefore, no evidence of IA was present in the majority of the included patients. On GM positive BALf samples, the resistance PCR could be successfully performed in 29 of 43 patients. In the 47 patients in which the resistance PCR was successful, the prevalence of RAMs was lower than expected at 8.5%. However, confidence intervals are wide so no definite conclusions can be drawn at this time. Also, with only 4 patients in whom a RAM was detected, no conclusions can be drawn on the impact of the PCR on patient outcome. Only 11 of the 130 patients (8%) were culture positive (9 of 35 (26%) of the GM positive patients). This underlines the importance of molecular methods to detect azole-resistance. The low rate of culture positive cases is not unexpected as it is in line with many other studies (11). However, only 1 of the 4 patients in whom azole-resistant IA was documented was culture negative. Remarkably, two of these patients had a co-infection with *Mucorales* and this is in line with a study by Pelzer *et al*. (14). In this single-centre study by Pelzer et al. the performance of the AsperGenius® was evaluated in 100 allogeneic stem cell recipients with pulmonary infiltrates undergoing BAL sampling (14). According to the EORTC/MSG criteria (15), 23 patients had probable IA, even though 11 patients had received azole prophylaxis. RAMs were documented in three patients (2 cases with TR34/L98H and 1 case with TR46/Y121F/T289A). All three cases were culture-positive and resistance was confirmed by classic phenotypic susceptibility testing. Remarkably, all three patients with azole-resistant IA were co-infected with *Mucorales*. *Aspergillus* PCR showed a sensitivity of 65% but combined with GM sensitivity and specificity was 96% and 100%, respectively.

An update of the Dutch guideline on the treatment of invasive fungal infections was published in December 2017 and tries to incorporate the fact that the prevalence of azole-resistance in the Netherlands was higher than 10% for several consecutive years as well as the observation that the initiation of inappropriate therapy leads to a statistically as well as clinically important increase in the overall mortality. (5,16). This Dutch guideline now recommends combination antifungal therapy (azole and echocandin or azole and liposomal-amphotericin B) as one of the treatment options for patients suspected of having IA at least until resistance has been ruled out by culture or molecular diagnostic methods. Treating all patients with non-azole antifungals like liposomal-

amphotericin B is associated with significantly more toxicity, is more expensive and can only be given intravenously. The latter is very cumbersome because treatment in immunocompromised haematology patients often needs to be given for months. With the results of this interim analysis, one may argue if combination antifungal therapy is actually necessary because resistance was observed in fewer than 10% of the patients in whom the resistance PCR was successfully performed. Furthermore, in the large majority of the patients in our study galactomannan and PCR were negative and therefore, IA was virtually excluded in the majority of patients. This means that starting combination therapy in all these patients would lead to a substantial overuse of non-azole antifungals as azole resistance could be documented in only 2% of them. Therefore, we recommend that, in general, antifungal therapy should not be initiated in patients who are planned to undergo bronchoscopy and BAL unless a very typical radiology is seen on high-resolution CT of the lungs or at the time when the GM (and/or PCR) turns out to be positive. If antifungal therapy is initiated preceding the bronchoscopy, the interim analysis of the AzoRMan study supports the alternative approach to patients with a suspected invasive aspergillosis mentioned in the Dutch guideline. This approach consists of starting azole monotherapy while waiting for prompt antifungal resistance testing by PCR and culture and adapting therapy according to the test results (17).

About one in four patients with a negative BAL GM, have a positive *Aspergillus species* PCR in this study. Due to lack of standardization, PCR was not yet included in the EORTC/MSG criteria of 2008 and it is not clear which patients with a positive PCR but a negative GM should be treated. In particular in those patients with atypical pulmonary infiltrates this is a difficult clinical decision to be made. In the 2019 update of the EORTC/MSG criteria, PCR has been included in the probable IA definition (18). Both tests are complementary but will not replace one another.

Unfortunately, this interim analysis also shows that the sample size of the AzoRMan study should be increased substantially if the primary endpoint of the study is to be answered. The estimated prevalence of resistance seems correct but in the sample size calculation we assumed that the resistance PCR would be successful in 75% of samples and that a higher proportion of the included patients would suffer from IA. Taking the results of the interim analysis into account, the sample size should be at least doubled to 600 patients.

CONCLUSION

The majority of the patients with a haematological disease that undergo BALf sampling to confirm or rule out an IA, do not have this infection. In the 47 patients in whom the resistance PCR was successful, the prevalence of CYP51A gene mutations was 8.5%.

Given the fact that in only 47 of the 212 patients included so far, the resistance PCR led to an interpretable result, the sample size of the study population needs to be increased substantially in order to answer the primary research question.

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Chapter 10.1

Invasive aspergillosis in patients admitted to ICU with severe influenza: A retrospective cohort study

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Teachers open the door, but you must enter by yourself. (Chinese wijsheid)

ABSTRACT

Background

Invasive pulmonary aspergillosis (IPA) typically occurs in an immunocompromised host. For almost a century, influenza has been known to set up for bacterial superinfections, but recently patients with severe influenza were also reported to develop IPA. We conducted a retrospective multicentre cohort study to measure the incidence of IPA over several seasons in ICU patients with influenza pneumonia and to evaluate whether influenza was an independent risk factor for IPA.

Methods

Data were collected from patients admitted to 7 ICUs with severe influenza during seven influenza seasons. To determine if influenza was independently associated with IPA, a subgroup of non-immunocompromised influenza-positive patients (cases) were compared with influenza-negative patients (controls) admitted to the ICU with community-acquired pneumonia (CAP) using logistic regression analyses.

Findings

Of the 432 patients admitted to the ICU with influenza, IPA was diagnosed in 19% (83/432) a median of 3 days after ICU admission. The incidence was comparable for influenza A and B. The incidence in the 117 immunocompromised influenza patients was as high as 32% (38/117), while 14% (45/315) of the non-immunocompromised influenza patients developed IPA. In contrast, only 5% (16/315) of the non-immunocompromised influenza-negative controls developed IPA ($p<0{\cdot}0001$). The 90-day mortality in influenza patients with and without IPA was 51% and 28%, respectively ($p<0{\cdot}0001$). In the retrospective cohort study, influenza was found to be independently associated with IPA (aOR 5·2, 95% CI 2·6-10·3, $p<0{\cdot}0001$), besides a higher APACHE II score, male sex and use of corticosteroids.

Interpretation

Influenza was identified as an independent risk factor for IPA and associated with a high mortality. Future studies should evaluate whether a faster diagnosis and/or antifungal prophylaxis could improve outcome of influenza-associated aspergillosis.

Funding

None

RESEARCH IN CONTEXT

Evidence before this study

We searched PubMed for articles published between January 1963 and October 2017, using the search terms “influenza” and “aspergillus” or “aspergillosis”. This search yielded case series which described invasive pulmonary aspergillosis (IPA) in patients admitted to the ICU with influenza. Yet, a systematic evaluation of the risk of IPA in a large population of ICU patients with influenza over several consecutive influenza seasons was missing. Also, it remained to be demonstrated if influenza was independently associated with aspergillosis.

Added value of this study

This study is, to our knowledge, the largest study ever performed on the risk for IPA in this patient population with 432 ICU patients with influenza included. It is also the first to evaluate this complication over several consecutive seasons in a large number of ICUs. Furthermore, by comparing non-immunocompromised influenza-positive and influenza-negative patients, we aimed to show that influenza was an independent risk factor for IPA. The following conclusions could be drawn: First, the incidence of IPA was >10% in each of the 7 seasons and was almost equal in influenza A and influenza B patients. Therefore, once a patient with influenza needs intensive care support, the risk for IPA does not depend on the influenza season and influenza subtype. Second, the overall incidence of aspergillosis was 19% and was as high as 32% in the subgroup of patients who were also immunocompromised at the time of their influenza infection. The overall mortality in the patients with IPA was very substantial at 51%. Last but not least, we compared 315 non-immunocompromised (i.e. no EORTC/MSG host factor) influenza-positive patients with an equal number of non-immunocompromised influenza-negative patients with severe community-acquired pneumonia (CAP) for the occurrence of IPA. We showed that influenza was independently associated with IPA (aOR 5·2, 95% CI 2·6-10·3, p<0·0001).

Implications of all the available evidence

The independent association between influenza and IPA and the high mortality calls for increased awareness and a more aggressive diagnostic approach. Future studies should evaluate if prophylaxis is useful.

INTRODUCTION

Invasive pulmonary aspergillosis (IPA) typically occurs in a severely immunocompromised host and isolation of *Aspergillus* species in the immunocompetent host is mostly considered colonization.[1,2] The six-week mortality of IPA is 20-30%[3,4] but is much higher in critically-ill patients.[4,5] Influenza is a common viral respiratory tract infection. In a subset of patients with influenza, intensive care admission is needed. This may be due to bacterial superinfection[1,6,7] but also influenza in itself can cause severe acute respiratory distress syndrome (ARDS),which is associated with a mortality of 14% to 41%.[8,9]

Influenza-associated aspergillosis was occasionally described decades ago and several small case series were reported recently.[1,9,10] 65% of the reported cases did not have classic host factors for IPA as defined by the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG).[1,11] These EORTC/MSG criteria are used to classify patients with a fungal infection into proven, probable or possible aspergillosis but are not applicable to the ICU setting. For the ICU setting, an algorithm (AspICU algorithm) was described by Blot and colleagues to distinguish IPA from *Aspergillus* colonization in critically-ill patients.[12]

In 2012, Wauters and colleagues reported an incidence of 23% of proven or probable IPA in 44 H1N1 influenza patients in two consecutive influenza seasons (2009-2011). Remarkably, 44% of the IPA cases lacked any of the classical EORTC/MSG host factors.[9] Recently, a Dutch study described 23 (16%) cases of IPA among 144 patients admitted to the ICU with influenza during the 2015-16 H1N1 influenza season.[13] These observations suggest that influenza infection requiring ICU admission is a risk factor for IPA and that the incorporation of influenza as a host factor in the current diagnostic criteria may be appropriate. However, it remains unclear if influenza is independently associated with the occurrence of IPA and if the risk varies from season to season. This study aims to describe the epidemiology and outcome of IPA in ICU patients over seven consecutive influenza seasons and to evaluate whether influenza is independently associated with IPA.

METHODS

Study design and data collection

We performed a retrospective cohort study in seven tertiary care ICUs (2 in Belgium and 5 in The Netherlands). The search strategy for influenza-positive patients admitted to the ICU during influenza seasons 2009-2016 consisted of reviewing all patients with a positive influenza polymerase chain reaction (PCR) in the registry of the local

microbiology department and matching these with ICU admissions. We selected a group of patients admitted to the ICU with severe community-acquired pneumonia (CAP) and with a documented negative influenza PCR test as the comparison group because these patients are equally admitted to the ICU from outside the hospital with respiratory insufficiency due to pneumonia as well (figure 1). A list of patients with a negative influenza PCR was retrieved from the microbiology departments and these patients were matched for ICU admission. All patients were evaluated whether an infiltrate was present on chest imaging, antibiotic therapy was initiated and if a diagnosis of CAP was made at ICU admission. The patient files were also reviewed to exclude that an influenza infection was diagnosed elsewhere and to confirm that the pneumonia was not hospital acquired. Figure 1 describes the inclusion process in detail. The study protocol was approved by the institutional review board (IRB) of both Belgian sites and by the IRB of the initiating Dutch centre (Erasmus University Medical Centre, Rotterdam) for the 5 Dutch sites.

Study population (figure 1)

Patients were ≥18 years, admitted to the ICU for >24 hours with acute respiratory failure, had pulmonary infiltrates on imaging and a confirmed influenza infection based on a positive airway PCR test. A subgroup of the influenza-positive cohort (cases) was compared with an influenza-negative comparison group (control group) for the occurrence of IPA. Cases were the subgroup of influenza-positive patients that did not have an EORTC/MSG host factor (table S1, appendix p3), already posing them at risk for IPA. Controls were patients admitted to the ICU for severe CAP with a negative influenza PCR. Like the cases, controls were EORTC/MSG host factor negative. Exclusion criteria for all patients were respiratory failure not being the primary reason for ICU admission and a history of IPA. Please note that the terms cases and controls do not point towards a case-control study design from a methodological point of view. They describe two patient groups where cases should be interpreted as “influenza-positive non-immunocompromised patients with respiratory insufficiency” and controls as “influenza-negative non-immunocompromised patients with CAP”.

Definition of Invasive Pulmonary Aspergillosis (IPA)

The definition of IPA is modified from the AspICU algorithm and is based on the presence of clinical, radiological and mycological criteria in all IPA cases.[12] Details on the definitions can be found in table 1.

This modified IPA definition does not require an EORTC defined host factor because otherwise patients with influenza but without an EORTC host factor could never fulfil the IPA definition as long as influenza is not part of the EORTC host factor definition. To be on the conservative side, we excluded all patients in whom the only mycological

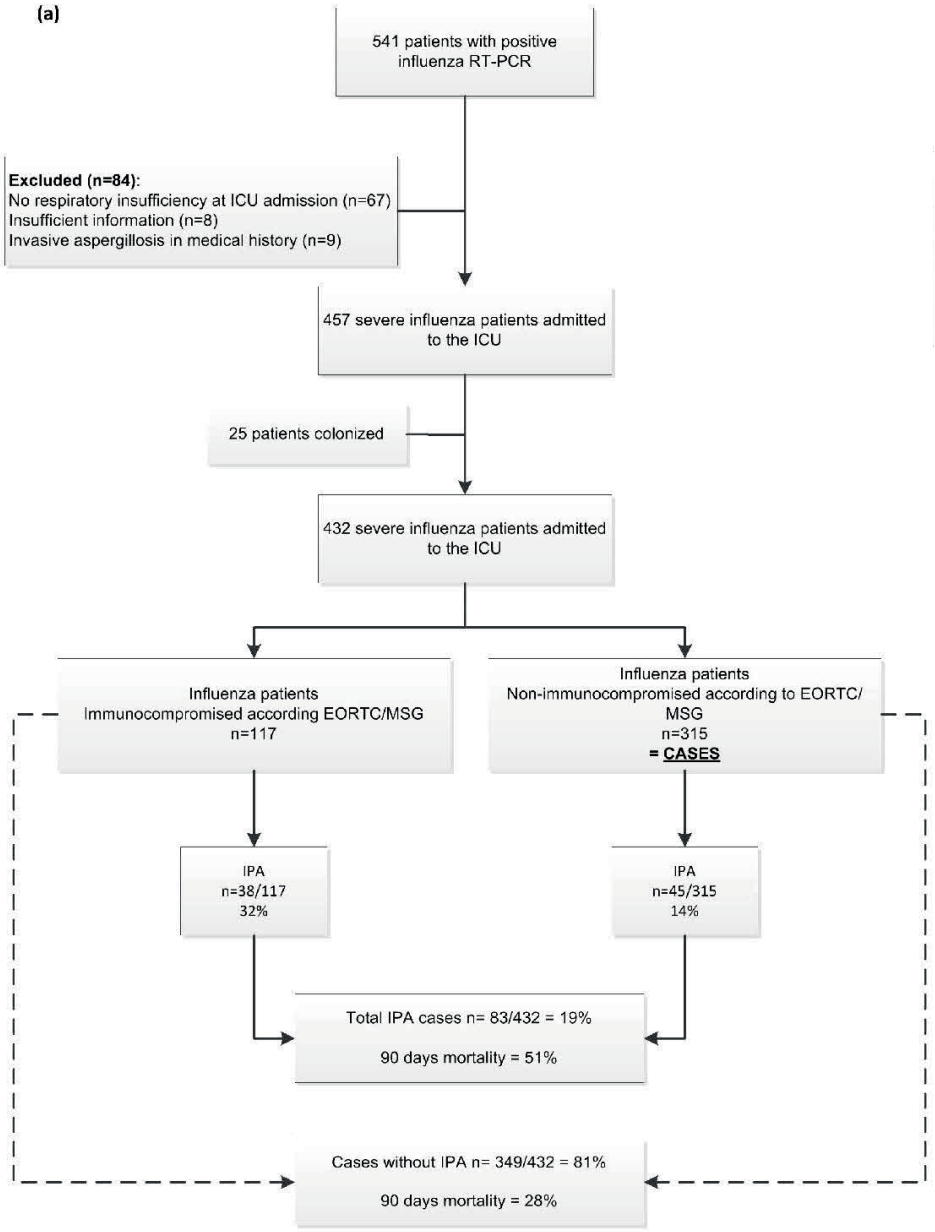
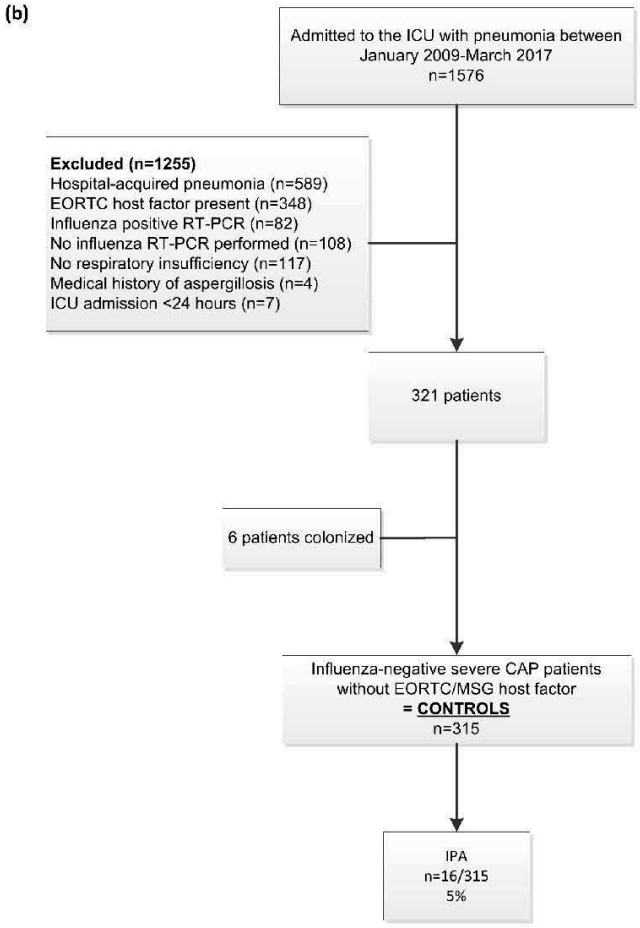


Figure 1: Overview of inclusion process and IPA cases with corresponding mortality: (a) inclusion process influenza patients and cases; (b) inclusion process control group.
Abbreviations: CAP= Community acquired pneumonia; EORTC/MSG= European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group; ICU= intensive care unit; IPA= Invsasive pulmonary aspergillosis; RT-PCR= real-time polymerase chain reaction



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evidence for IPA was a lower positive respiratory tract culture (sputum, bronchial aspirate) for *Aspergillus* species but who had a negative or unavailable broncho-alveolar lavage (BAL) culture and galactomannan test. These patients were defined as colonized and were excluded from the final analysis.[14] Every influenza patient was reviewed and consensus was achieved (NP,RV,AS,LV,JW,BR) to ascertain whether the modified IPA definition was met.

MODIFIED IPA DEFINITION: The definition of IPA is modified from the *AspICU* algorithm and is based on the presence of clinical, radiological and mycological criteria in all IPA-cases.
CLINICAL CRITERIA: *one of the following signs/symptoms has to be present:* • **Fever refractory to at least 3 days of appropriate antibiotic therapy** • **Recrudescent fever after a period of defeverescence of at least 48 hours while still on antibiotics and without other apparent cause** • **Dyspnea** • **Hemoptysis** • **Pleural friction rub/chest pain** • **Worsening respiratory insufficiency in spite of appropriate antibiotic therapy and ventilatory support**
RADIOLOGICAL CRITERIA: any infiltrate on pulmonary imaging by portable chest X-ray or CT-scan of the lungs. This radiological definition is different from the EORTC defined radiological criteria (e.g. halo sign or air-crescent sign) because these EORTC criteria apply to patients with prolonged neutropenia but are of little use for ICU patients.
MYCOLOGICAL CRITERIA: *≥1 of the following has to be present* • **Histopathology or direct microscopic evidence of dichotomous septate hyphae with positive culture for *Aspergillus* from tissue** • **A positive *Aspergillus* culture from a broncho-alveolar lavage (BAL)** • **A galactomannan optical index on BAL of ≥1** • **A galactomannan optical index on serum of ≥ 0.5.**

Table 1: Modified IPA definition
The Platelia Aspergillus test was used for galactomannan detection in all centers (Bio-Rad Laboratories, Marnes-la-Coquette, France). *Aspergillus* species were identified by their culture characteristics and microscopic morphology.

STATISTICAL ANALYSIS

In univariable analysis, categorical variables were compared by Fisher's exact test and Chi-square test, and continuous variables with t-test or Mann-Whitney U test where appropriate. On the entire population of the influenza-positive cohort, a multivariable analysis was done by binary logistic regression to detect independent risk factors for the development of IPA in influenza patients. The dependent variable was the presence of IPA and independent variables were those previously described as a possible risk factor for IPA in the ICU or associated with IPA in the univariable analysis.[4,15] The estimate of association was expressed as adjusted odds ratio (aOR) with corresponding

95% confidence interval (CI). Multiple imputations were performed to handle missing data, using 20 imputations and 1000 iterations following the Markov-Chain Monte Carlo methods, and the pooled results are presented (Table S4, appendix p5). In addition, a binary logistic regression analysis with multiple imputations was performed on the entire cohort of cases and controls to determine if influenza was independently associated with IPA. Data were analysed with SPSS Version 24 (Armonk, NY:IBM corp). No correction for multiple testing was performed for the univariable analyses and a two-tailed significance level of 0·05 was used. These p-values should therefore be interpreted with this limitation in mind. A statistician from the department of Biostatistics of Erasmus University Medical Center (ERA) supervised the analysis.

Role of funding source

This study was part as part of our routine work. No funding was provided. The corresponding author had full access to all of the data and the final responsibility to submit for publication.

RESULTS

Inclusion/exclusion process (figure 1)

Between 01/2009 and 06/2016, 541 influenza-positive patients were admitted to 7 ICUs. 84 patients were excluded for the following reasons: respiratory insufficiency was not the reason for ICU admission (n=67), medical history of IPA (n=9) or insufficient clinical data (n=8). Another 25 patients were excluded because they met the criteria for *Aspergillus* colonization. In total, 432 patients with influenza were included in the influenza cohort. 315 of them were EORTC/MSG host factor negative and were defined as cases. The search strategy for the comparison group with severe CAP patients resulted in the selection of 315 patients (figure 1).

Influenza patient characteristics (table 2)

Patient characteristics are summarized in table 2. Mean age was 59 years and 56% (240/432) were male. Influenza A and B was found in 82% (355/432) and 18% (77/432), respectively. 79% (338/432) of the patients received a neuraminidase inhibitor. 27% (117/432) were EORTC/MSG host factor positive. The mean APACHE II score was 22 SD±8. 75% (326/432) of the patients required intubation for mechanical ventilation for a median duration of 11 [IQR 5,21] days. 52 patients received extracorporeal membrane oxygenation (ECMO). The overall ICU mortality in the influenza-positive patients was 25% (107/432).

	Influenza cohort (*n* = 432)	IPA (*n* = 83)	No IPA (*n*=349)	*p*
BASELINE FACTORS				
Age (years)	59 ± 15	60 ± 12	59 ± 16	0·35
Sex (men), *n (%)*	240 (56)	56 (67)	184 (53)	0·015*
APACHE II score on admission	22 ± 8	25 ± 9	22 ± 7	0·005*
BMI >30 kg/m^2, *n (%)*	93/410 (23)	17/83 (20)	76/327 (23)	0·59
Diabetes, *n (%)*	88 (20)	10 (12)	78 (22)	0·036*
Liver cirrhosis	25 (6)	5 (6)	20 (6)	1·0
Chronic kidney disease	71 (16)	16 (19)	55 (16)	0·44
KNOWN RISK FACTORS				
EORTC host factor, *n (%)*	117 (27)	38 (46)	79 (23)	<0·0001*
Haematological malignancy, *n (%)*	66 (15)	22 (27)	44 (13)	0·002*
Solid organ transplant, *n (%)*	32 (7)	11 (13)	21 (6)	0·024*
Solid organ malignancy, *n (%)*	21 (5)	4 (5)	17 (5)	1·0
Neutropenia, *n (%)*	22 (5%)	11 (13)	11 (3)	0·001*
COPD, *n (%)*	79 (18)	13 (16)	66 (19)	0·49
STUDIED RISK FACTORS				
CS 28 days before ICU, *n (%)*[a]	145/426 (34)	46/82 (56)	99/344 (29)	<0·0001*
Median dose CS 28days before ICUadm (IQR)	0·14 (0·06;0·28)	0·22 (0·10;0·33)	0·10(0·06;0·24)	0·003*
Smoking in the past year	114/332 (34)	26/61 (43)	88/271 (32)	0·13
ICU DATA				
Mechanical ventilation, *n (%)*	326 (75)	75 (90)	251 (72)	0·0004*
Mechanical ventilation days (IQR)	11 (5,21)	14 (9,31)	9 (4,17)	0·001*
NO/HFOV, *n (%)*	42 (10)	13 (16)	29 (8)	0·04*
ECMO or ECCOR, *n (%)*	52 (12)	16 (19)	36 (10)	0·024*
Vasopressors, *n (%)*	287/423 (67)	67/82 (81)	220/341 (65)	0·002*
RRT, *n (%)*	100/423 (24)	35/83 (42)	65/340 (19)	<0·0001*
Median days of ICU stay [IQR]	11 [6,23]	19 [12,38]	9 [5,20]	<0·0001*
OUTCOME DATA				
ICU mortality, *n (%)*	107 (25)	37 (45)	70 (20)	<0·0001*
Hospital mortality, *n (%)*	133 (31)	41 (49)	92 (26)	<0·0001*
90 days mortality after ICU admission, *n (%)*	141 (33)	42 (51)	99 (28)	0·0001*
INFLUENZA				
Influenza A, *n (%)*	355 (82)	71 (86)	284 (81)	0·37
Influenza B, *n (%)*	77 (18)	12 (14)	65 (19)	0·37
Influenza treatment, *n (%)*	338/428 (79)	70/83 (84)	268/345 (78)	0·25
DIAGNOSTICS				
BAL sampling performed, *n (%)*	233 (54)	81 (98)	152 (44)	<0·0001*
BAL culture positive, *n (%)*		50/80 (62·5)		
BAL Galactomannan performed, *n(%)*	137 (32)	76 (92)	61 (17)	<0·0001*
BAL Galactomannan positive, *n (%)*		67/76 (88)		
Serum GM serum performed, *n (%)*	47 (11)	31/83 (37)	16 (5)	<0·0001*

Table 2: Overview of the characteristics of influenza-positive cohort
APACHE= acute physiology and chronic evaluation score, BAL= broncho-alveolar lavage, BMI= body mass index, COPD= chronic obstructive pulmonary disease, CS= corticosteroids, ECMO= extracorporeal membrane oxygenation, EORTC= European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, GM= galactomannan; ICU= intensive care unit, IPA= invasive pulmonary aspergillosis, IQR= interquartile range, NO/HFOV= nitric oxide/high-frequency oscillation ventilation, RRT= renal replacement therapy, SD= standard deviation.

IPA characteristics in patients with Influenza (table 3)

83 (19%) of the severe influenza patients admitted to the ICU fulfilled the IPA definition. The proportion of patients with IPA among the influenza cases varied per centre (6% to 26%, table S2, appendix p3). IPA was diagnosed at a median of 3 (IQR 0,7) days after ICU admission. *A. fumigatus* was almost exclusively cultured when identification to the species level was available. Susceptibility data were available in 17 patients and 4 voriconazole-resistant strains were documented. While the number of patients admitted to the ICU with influenza varied substantially from year to year, the prevalence of IPA was >10% in all calendar years (figure S4, see appendix p5). IPA was found in 20% (71/355) and 16% (12/77) from the patients with influenza A and influenza B pneumonia, respectively. No clear association could be demonstrated between the prevalence of IPA and the influenza subtypes that circulated in the respective calendar years (table S6, appendix p6).

In 98% of the IPA cases (81/83) a BAL was done, yielding a positive Aspergillus culture in 50 (60%) and a positive galactomannan test (optical density (OD) ≥1·0) in 67 (88%) of the 76 patients of whom the BAL was not only cultured but also tested for the presence of galactomannan (table 3). Serum galactomannan test was done in 31/83 (37%) of the IPA cases and was positive (i.e.≥0·5) in 65% (20/31). 21 of the 83 patients (25%) with IPA were previously healthy and 7 (33%) of them died. Given the fact that by definition patients with influenza who are not immunocompromised do not fulfil the EORTC/MSG host factor definition, only 36 of 83 (43%) had a proven (n=16) or probable (n=20) IPA according to the EORTC/MSG classification.[11] According to the AspICU algorithm, specifically designed for ICU patients, 48 patients (58%) were diagnosed with IPA while 30 were not classifiable as they had a positive galactomannan test on BAL but a negative lower respiratory tract culture which is the entry criterion in the AspICU algorithm.[12] 92% of the IPA cases (76/83) received mould-active antifungal therapy. In these patients, no difference in the number of days from influenza diagnosis to antifungal therapy initiation was observed between survivors and non-survivors 90 days after ICU admission (4 [IQR 1,10] versus 5 [IQR 1,7] days, p=0·64).

IPA characteristics in patients with Influenza	
BAL culture positive, *n (%)*	50/80 (62·5)
BAL Galactomannan positive, *n (%)*	67/76 (88)
Serum Galactomannan positive, *n (%)*	20/31 (65)
EORTC/MSG criteria	
• **Proven, *n (%)***	16 (19)
• **Probable, *n (%)***	20 (24)
• **Not classifiable, *n (%)***	47 (57)
AspICU criteria	
• **Proven, *n (%)***	16 (19)
• **Putative, *n (%)***	32 (39)
• **Colonizer, *n (%)***	5 (6)
• **Not classifiable, *n (%)***	30 (36)
Initial Treatment:	
• **Voriconazole, *n (%)***	61 (73)
• **Echinocandine, *n (%)***	2 (2)
• **Combination (Triazole + Echinocandine), *n (%)***	9 (11)
• **Liposomal-Amfotericine B, *n (%)***	4 (5)
• **No treatment, *n (%)***	7 (8)

Table 3: IPA characteristics in patients with Influenza
BAL= broncho-alveolar lavage, EORTC/MSG= European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, IPA= invasive pulmonary aspergillosis.

Comparison of influenza patients with and without IPA (Table 2)

ICU mortality in the IPA cases was higher than in patients without IPA (37/83 or 45% versus 70/349 or 20%, p<0·0001) and the ICU stay was longer (19 days [IQR 12,38] versus 9 days [IQR 5,20], p<0·0001). The mortality 90 days after ICU admission was 51% (42/83) in patients with IPA and 28% (99/349) in those without IPA (p<0·0001).

Patients with IPA required mechanical ventilation more often (90% (75/83) versus 72% (251/349), p=0·0004) and for a longer period (+5 days; p=0·001).

Independent risk factors for the occurrence of IPA on the pooled data of all influenza-positive patients (regardless of the presence or absence of EORTC/MSG host factor) are presented in figure 2a. A list of all variables used in the multivariate analyses can be found in the appendix (table S4, appendix p5). Corticosteroid (CS) therapy in the 4 weeks before ICU admission was independently associated with IPA (aOR 1·59, 95% CI 1·30 to 1·99;p<0·0001) per 0·1mg/kg/day prednisone equivalent). Finally, male sex (aOR 1·84, 95% CI 1·05 to 3·22;p=0·034) and a higher admission APACHE II were associated with IPA as well (aOR 1·05,95% CI 1·01 to 1·09;p=0·007 per 1·0 point APACHE II increase).

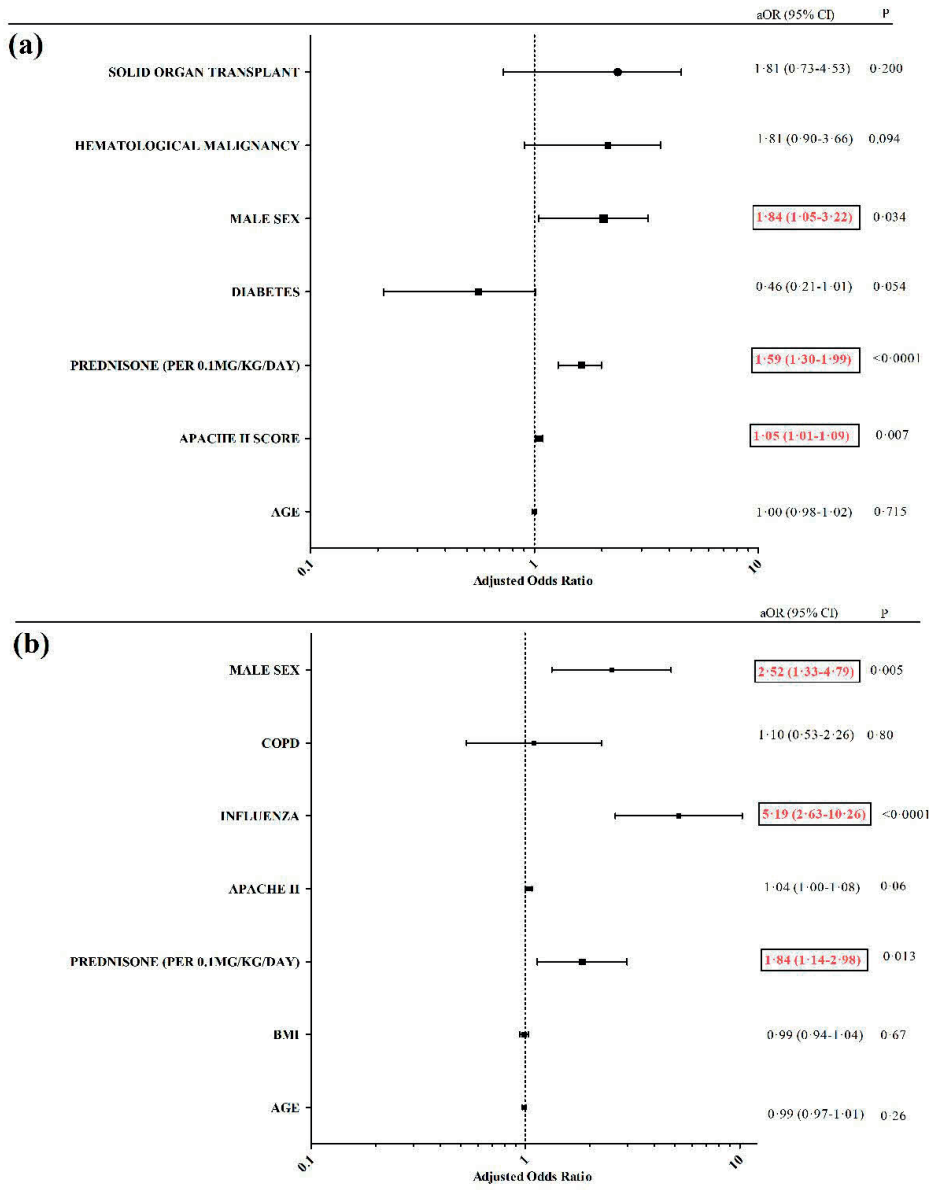

Figure 2: Forest plots of risk factors for the development of IPA
The solid lines represent the 95% confidence intervals (CIs). There has been corrected for centre as well but this is not depicted here as no significant differences were found. (a) Analysis of risk factors for patients with influenza in the ICU to develop IPA. (b) Overview of case-control comparison. Factors independently associated with the development of IPA are highlighted in red.
Abbreviations: APACHE= acute physiology and chronic evaluation score; BMI= body mass index; CI= confidence interval; COPD= chronic obstructive pulmonary disease.

	All EORTC neg patients (n=630)	Influenza + CASES (n=315)	Influenza - CONTROLS (n=315)	*p*
BASELINE CHARACTERISTICS				
Age (years)	59 ± 17	58 ± 16	60 ± 17	0·15
Sex (men), ***n (%)***	371 (59)	169 (54)	202 (64)	0·008*
APACHE II admission	23 ± 8	22 ± 8	23 ± 8	0·29
BMI (kg/m^2), median (IQR) Missing, n	25 (22,29) 21	27 (23,30) 18	24 (22,28) 3	<0·0001*
Diabetes, ***n (%)***	114 (19)	63 (20)	51 (16)	0·21
Liver cirrhosis, ***n (%)***	44 (7)	18 (6)	26 (8)	0·21
Chronic Kidney Disease^x,***n (%)***	69 (11)	31 (10)	38 (12)	0·37
COPD, ***n (%)***	123 (20)	68 (22)	55 (17)	0·19
CORTICOSTEROIDS				
CS 28 days before ICU, ***n (%)***	99/619 (16)	57/304 (19)	42/315 (13)	0·005
Median dose CS 28days before ICUadm (IQR) when receiving CS	·078 [·054, ·176]	·070 [·054, ·171]	·080 [·053, ·179]	0·79
Missing, n	22	10	12	
ICU PARAMETERS				
Mechanical ventilation, ***n (%)***	475 (75)	246 (78)	229 (73)	0·12
Median ventilation days, days [IQR] Missing, n	9 [4, 18] 35	11 [5, 21] 26	4 [4, 14] 9	0·002*
NO/HFOV, ***n (%)***	64 (10)	37 (12)	27 (9)	0·17
ECMO or ECCOR, ***n (%)***	65 (10)	45 (14)	20 (6)	0·04*
ECMO days, median [IQR]	10 [6, 20]	11 [8, 21]	9 [5, 18]	0·44
Vasopressors, ***n (%)***	415 (66)	216 (69)	199 (63)	0·17
Renal replacement therapy, n(%)	103 (16)	61/307 (20)	42 (13)	0·03*
OUTCOME DATA				
ICU mortality, ***n (%)***	125 (20)	58 (18)	67 (21)	0·37
Hospital mortality, ***n (%)***	164 (26)	76 (24)	88 (28)	0·28
Mortality< 90 days after ICU admission	177 (28)	78 (25)	99 (31)	0·70
Median ICU stay, days [IQR] Missing	11 [6, 21] 19	11 [6, 23] 15	10 [6, 18] 4	0·15
IPA, ***n (%)***	61 (10)	45 (14)	16 (5)	<0·0001*
DIAGNOSTICS				
BAL sampling, ***n (%)***	318 (50)	145 (46)	173 (55)	0·026*
BAL Galactomannan performed, ***n(%)***	187 (30)	81 (26)	106 (34)	0·029*
IPA AspICU CLASSIFICATION				
IPA-proven, *n (% of IPA cases)*	8 (13)	6 (13)	2 (13)	
IPA-putative, *n (% of IPA cases)*	32 (52)	27 (60)	5 (31)	
IPA-colonizer, *n (% of IPA cases)*	4 (7)	3 (7)	1 (6)	
IPA non classifiable, *n (% of IPA cases)*	17 (28)	9 (20)	8 (50)	

Table 4: Overview of characteristics of the cases and controls

APACHE= acute physiology and chronic evaluation score, AspICU: algorithm for invasive aspergillosis in ICU as described by Blot and colleagues[12], BAL= broncho-alveolar lavage, BMI= body mass index, COPD= chronic obstructive pulmonary disease, CS= corticosteroids, ECCOR= extracorporeal CO2 removal, ECMO= extracorporeal membrane oxygenation, adm= admission, EORTC= European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, ICU= intensive care unit, IPA= invasive pulmonary aspergillosis, IQR=Interquartile range, NO/HFOV= nitric oxide/high-frequency oscillation ventilation, RRT= renal replacement therapy, SD= standard deviation. X=Glomerular filtration rate < 60mg /1.73m2

Comparison of influenza-positive cases and influenza-negative CAP controls (Table 4 and association analysis figure 2b)

315 of the influenza patients had no underlying EORTC/MSG risk factor and were defined as cases and 45 (14%) of them were diagnosed with IPA. In comparison, 16 of the 315 CAP controls (5%) were diagnosed with IPA. Baseline characteristics of the cases and controls are summarized in table 4. BAL sampling and galactomannan measurement on BAL was more frequently performed in CAP controls (BAL in 55% (173/315), galactomannan in 34% (106/315)) than in influenza-positive cases (46% (145/315) and 26%(81/315)). To evaluate if in the pooled patient population of influenza cases and CAP controls, influenza was associated with IPA, a binary logistic regression analysis was performed. This analysis confirmed the independent association between influenza and IPA (aOR 5·19 95% CI, 2·63 to 10·26; p<0·0001) (figure 2b). A list of all variables used in the multivariate analyses can be found in the appendix (table S5, appendix p5). In the case-control analyses, other independent risk factors for IPA were male sex and receipt of corticosteroids in the 4 weeks preceding ICU admission at a dose below the corticosteroid dose that is included in the EORTC/MSG host factor definition (figure 2b).

DISCUSSION

To the best of our knowledge, this study is the largest ever performed on the incidence, risk factors and outcome of IPA in ICU patients with influenza. Furthermore, the data provide evidence that influenza infection is an independent risk factor for IPA. Indeed, in a total of 630 non-immunocompromised patients admitted to the ICU with CAP of which 50% were infected with influenza, the presence of influenza increased the risk of IPA from 5 to 14%. Furthermore, the ICU mortality in the IPA cases was 45% and even in previously healthy individuals the mortality was 33%. This is in accordance with the 47% mortality described in earlier case series[1] but somewhat lower than described in recent cohorts.[13,16] Of note, 85 patients of this cohort had been included in previous studies.[9,13] In the subgroup with an EORTC/MSG host factor, the IPA incidence was as high as 32% and 61% of them had died 90 days after ICU admission. As the diagnosis of

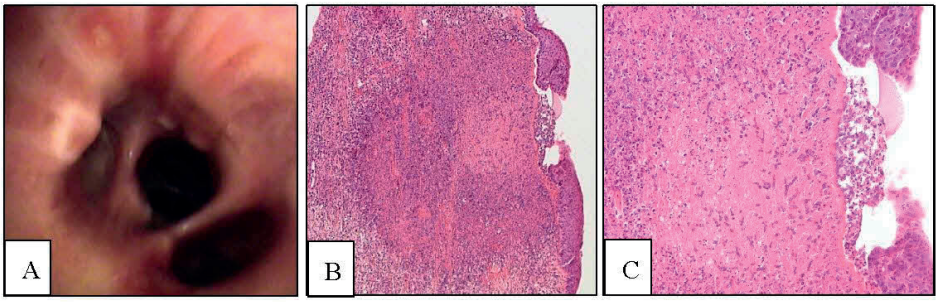

Figure 3: Bronchoscopy and histopathology of a representative case of influenza-associated ***Aspergillus*** tracheobronchitis.

A. Bronchoscopic examination reveals diffusedly inflamed mucosal tissue, with multiple whitish nodules, dispersed from trachea into main bronchial structures. Some nodules show central necrosis.

B. Haematoxylin-Eosin staining of biopsied specimen from trachea at 50x magnification, showing focal ulceration with submucosal necrosis and squamous metaplasia.

C. Haematoxylin-Eosin staining of biopsied specimen at 100x magnification, revealing invasion of submucosa by fungal hyphae, type *Aspergillus*, and dense infiltration with neutrophils.

IPA is challenging, a systematic approach towards the diagnosis of IPA in ICU patients with influenza may result in an even higher incidence of IPA and should be the focus of future prospective studies.

The reported overall incidence of IPA in critically ill patients varies widely from <1 to 6·9%[15,17,18] and corresponds with the 5% incidence in our CAP control group.[9,13,19] A recent study of 2901 ICU patients with influenza showed the presence of a co-infection in 17% and *Aspergillus* spp. in 7% of these patients with a co-infection.[20] The lower incidence in this study could be explained by a different diagnostic approach (e.g. no use of BAL galactomannan measurement), a lower overall awareness as well as the fact that only co-infections diagnosed within two days of hospital admission were registered.

As influenza is not considered a host factor for IPA, only part of our patients with IPA fulfilled one of the diagnostic criteria for IPA as defined by the EORTC/MSG or AspICU algorithm.[11,12] In addition, influenza patients with IPA mostly have non-specific radiology and classic radiological features only occur in 5% of critically-ill patients with IPA.[1,12,14,21,22] Autopsy series indicated that strict interpretation of the host factors for invasive fungal disease contributes to missed diagnosis of IPA.[5,23] Therefore, we classified our patients using a modified IPA definition in which no specific host factor was required. However, stringent mycological criteria were used, compatible with the case definition of EORTC/MSG, AspICU and van de Veerdonk and colleagues.[11-13] Of course, the same classification was used for the control group. Furthermore, to avoid an overestimation of the incidence of IPA, we excluded all 25 patients with only a positive

respiratory tract (i.e. sputum or tracheal aspirate) but a negative or unavailable BAL culture as the only microbiological evidence for IPA.

The optical density (OD) cut-off above which a galactomannan test in BAL should be considered positive is a matter of debate. The sensitivity of BAL galactomannan measurement was 88% when applying an OD≥0·5 on biopsy proven IPA cases in the ICU.[24] However, in a recent observational study the value of BAL galactomannan testing in the ICU was questioned because the specificity compared with a positive BAL culture was low at 38% and 62% with a galactomannan OD cut-off of ≥1·0 and 3.0 respectively.[25] However, given the limited sensitivity of BAL culture for the diagnosis of IPA, the use of a positive culture as the gold standard makes the interpretation of their results difficult. In our case definition we used a galactomannan OD cut-off of ≥1·0. Yet, when an OD≥3·0 would be applied only 8 (10%) of the 83 IPA-influenza cases would have been classified differently. In addition, the median galactomannan OD of all influenza-IPA cases was as high as 5·8 IQR [2·8-6·7]. Furthermore, when we reviewed all 15 patients with proven IPA that also underwent BAL galactomannan testing, in 14 of these 15 patients with biopsy proven IPA, the BAL GM optical density was >1.0. Also, 6 patients without IPA had a galactomannan BAL measurement with a value <1.0 and were autopsied. In none of them, an IA was found at autopsy. Therefore, the specificity of galactomannan in BAL with a cut-off threshold of 1.0 in our study seems to be excellent. Remarkably, the observation that 17 of the 28 patients (61%) with a positive BAL galactomannan test, also had a positive serum GM was unexpected as in non-neutropenic haematology patients the sensitivity of serum galactomannan is low. This suggests that angio-invasion is often present in influenza patients with IPA.

We could not confirm the previous observation that a delayed initiation of anti-fungal therapy was associated with a fatal outcome.[13] A particularly high awareness was present in one of the participating centres as this centre already published on influenza-associated IPA in 2012. In this centre, BAL sampling was performed in 102 of 149 influenza patients. 26% of the patients in this centre fulfilled the IPA diagnosis with an ICU mortality of 38% compared to an ICU mortality of influenza-associated aspergillosis in all other centres of 50%. This suggests that increased awareness may improve outcome.

Azole resistance is an emerging problem and has been particularly reported in The Netherlands with a prevalence of 13% in 2016.[26] As azole resistance testing has only recently become a standard procedure in ICU, data on azole resistance were available for 17 patients only and resistance was documented in 4 of them.

Why patients with influenza are at risk for IPA is not yet clear.[27,28] Respiratory epithelium damage and mucociliary clearance dysfunction may facilitate the invasion of *Aspergillus* (figure 3).[7,9,29] Moreover, influenza-induced ARDS and hypoxia may cause immune-paralysis.[30-32] Almost all cases to date have been associated with the pandemic

influenza A H1N1 infection but influenza B may trigger an *Aspergillus* superinfection as well.[13,33] This observation was confirmed in this study as an almost equal proportion of both influenza A and influenza B patients developed IPA. We were unable to look at the influenza subtype as a possible risk factor for IPA as subtyping was only available in a small number of patients. However, no association could be found between the of IPA and the influenza subtypes that circulated in the respective calendar years. Furthermore, the fact that the of IPA was >10% in all calendar years suggests that the severity of illness rather than influenza subtype is more important. Whether our observation is specific for influenza or if it may also apply to pneumonia patients admitted to the ICU with a respiratory virus other than influenza remains to be seen. The observation that the use of CS prior to the ICU admission was independently associated with IPA is in accordance with a Cochrane review showing an association between CS use and increased influenza mortality. On the other hand, CS use before ICU admission could be a marker of the severity of the influenza infection, making it a possible confounder by indication. However, the available evidence on the value of CS in patients with influenza argues against its use as long as data from a prospective randomized clinical trial are lacking.[34]

Given the high incidence of IPA we observed, antifungal prophylaxis might be a valid approach. Whether antifungal prophylaxis will be superior to a standardized diagnostic approach combined with prompt initiation of antifungal therapy as soon as IPA is diagnosed remains to be demonstrated.

Limitations

First, given the retrospective design of this study, confounding cannot be ruled out and a standardized diagnostic approach towards IPA was lacking. However, the time needed to collect a similar amount of data prospectively clearly argues for the added value of this retrospective study. Also, as we did not correct for multiple testing, all univariate p-values should be interpreted with this in mind. Second, as only 60% of the patients with IPA had a positive BAL culture, the diagnosis of IPA was based on a positive galactomannan BAL test in a substantial number of patients. Given the observation that BAL sampling was performed in 98% of the influenza patients diagnosed with IPA but only in 44% of the patients not diagnosed with IPA we cannot exclude that the actual incidence of IPA may be even higher. We have no reasons to believe that compared with the influenza patients, a risk of underdiagnosis of IPA in the influenza-negative controls was present. Actually, BAL galactomannan sampling was more often performed in our control group. Third, one may argue that in a subset of the patients the IPA will have developed before ICU admission and may have resulted in clinical deterioration and ultimately ICU admission. However, this does not change the conclusion that in patients with influenza that need ICU support, IPA is highly prevalent and associated with a high mortality. Another limitation is that all but 1 of the 7 centres were tertiary

care academic ICUs. Therefore, extrapolation to small primary care ICUs should be done with caution. However, the incidence of IPA in the single primary care ICU of this study was comparable at 15%. The use of ECMO support was somewhat higher in the influenza-positive cases (14%) than in the influenza-negative CAP controls (6%) and therefore one may argue that ECMO may be a confounder in the analysis. However, only 4 of 83 IPA infections in our study were diagnosed with IPA >72h after the start of ECMO support. Also, in a recent study on fungal infections in 2129 patients on ECMO the incidence of *Aspergillus* superinfections was similar to the general intensive care population. Importantly, this study confirmed that in the subgroup of influenza patients on ECMO, the incidence of IPA was 14%.[35] A final limitation is the choice of our comparison group. By choosing severe CAP patients as controls we can only conclude that compared with influenza-negative patients with CAP, the presence of influenza is a risk factor for IPA. Several other patient groups could have been chosen as controls as well (e.g. non-infectious ARDS) but we preferred a control group that was comparable to the influenza cases as much as possible. Therefore, we considered patients with CAP the most appropriate controls as, like influenza patients, they present with acute respiratory failure and are admitted to the ICU from the community.

CONCLUSION

In ICU patients with influenza, the incidence of IPA was high as was the mortality. Influenza was independently associated with IPA. An aggressive diagnostic approach should be pursued and the value of antifungal prophylaxis studied.

Contributors

Study design: A.F.A.D.S., J.W., B.J.A.R.; information technology and database: N.P., R.V., A.F.A.D.S.; data preparation: all authors; data collection: N.P., R.V., L.V., A.F.A.D.S; data analysis: N.P., J.W. R.V., B.J.A.R., A.F.A.D.S.; statistical analysis: E.R.A., R.V., A.F.A.D.S.; writing of first draft: A.F.A.D.S, J.W., B.J.A.R; revision of manuscript: all authors.

Declaration of interests

BJAR received research grants from Gilead and MSD outside the context of this study. He also received travel grants from MSD, Gilead, BMS, Jansen-Cilag, ViiV and Abbvie and received personal fees from Gilead, Viiv and Great-Lake pharmaceuticals. He served as an advisor to Gilead, ViiV, BMS, Abbvie, Jansen-Cilag and MSD. AFADS received travel grants to attend international conferences from Gilead, Pfizer and Roche outside the context of this study. JW received research grants from Pfizer and MSD outside the

context of this study. He also received travel grants from MSD, Gilead and Pfizer. KL received research grants from Pfizer, Gilead and MSD outside the context of this study. She also received travel grants from MSD, Gilead and Pfizer and served as an advisor for Pfizer and MSD. PEV reports research grants from F2G, MSD, Gilead Sciences and non-financial support from OLM diagnostics and IMMY diagnostics outside the context of this study. All other authors: none to declare.

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Chapter 10.2

Influenza-associated pulmonary aspergillosis: a local or a global lethal combination?

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Al wat ik weet is dat ik niets weet. (Socrates)

EDITORIAL

For almost a century, superinfections with S. *pneumoniae* and S. *aureus* have been a well-known complication of seasonal influenza. More recently, invasive pulmonary aspergillosis (IPA) was described as another important complication. Influenza associated invasive pulmonary aspergillosis (IAPA) has so far been predominantly described in critically ill patients admitted to the ICU with influenza pneumonia [1-3]. Following a number of single-center case series of IAPA, the Dutch-Belgian Mycoses Study Group (DB-MSG) evaluated its incidence in the largest cohort study of patients admitted to the ICU with influenza so far. In this study, 19% of the 432 patients admitted to the ICU during seven consecutive influenza seasons were diagnosed with IAPA. Furthermore, the study also demonstrated that in patients admitted to the ICU with community-acquired pneumonia, the detection of influenza was strongly associated with a subsequent diagnosis of invasive pulmonary aspergillosis and half of the patients diagnosed with IAPA died in the ICU [1].

In this issue of *Clinical Infectious Diseases*, a single-center retrospective cohort study performed over five consecutive influenza seasons at a large tertiary care center in Alberta, Canada reports on the incidence of IPA in 111 patients admitted to the ICU for respiratory failure caused by an influenza infection. These data are a welcome addition to the data currently available in the literature. In contrast with the incidence of IAPA of 12 to 28% described in Europe and Asia so far, Schwartz *et al* diagnosed an IAPA in only 8 of 111(7.2%) patients. Before we start wondering about how it is possible that the incidence of IAPA in Canada may be lower than in Europe or Asia, it is important to put this incidence of 7% into perspective. Indeed, apart from patients undergoing remission induction chemotherapy for acute myeloid leukemia, patients with severe graft-versus-host disease and perhaps also lung transplant patients, no other patient population has an incidence of IA as high as 7%.

Histopathological evidence of the presence of *Aspergillus species* from a sterile body site remains the gold standard of IPA diagnosis. However, sampling lung tissue in an ICU patient is clearly not without risk and actually rarely performed. Sputum or tracheal aspirate cultures are a low-cost and easy to perform diagnostic test but the sensitivity when used to diagnose IPA in ICU patients does not exceed 50% [4]. Several non-culture-based assays are now available to demonstrate the presence of *Aspergillus* in blood or airway samples and testing for the presence of galactomannan (GM), a cell wall component of *Aspergillus*, is the most-validated of these non-culture based tests. Because most studies that evaluated GM for the diagnosis of IA in ICU patients included few patients with a proven infection, doubts remain regarding the value of GM testing in ICU patients. However, in a prospective study that was conducted in the setting of a very high autopsy rate a substantial number of proven infections were included. In

this unique study, testing for the presence of GM on broncho-alveolar lavage (BAL) fluid had a sensitivity 88% and specificity of 87%. One of the most striking observations in this study was that GM testing on BAL identified 11 of a total of 26 (autopsy) proven IPA cases. Without GM testing these cases would have been missed if fungal cultures had been used only. As expected, GM testing on serum performed substantially poorer[5, 6]. In the study by Schwartz and colleagues, clinicians tested for the presence of GM on BAL in as few as 16 of the 111 patients. It is therefore very likely that the incidence of 7% would have been higher if GM had been tested on BAL in all patients.

However, a true difference in incidence of IAPA across continents may well be the case and several hypotheses can be postulated here. Differences in the incidence of invasive aspergillosis have been linked to single nucleotide polymorphisms in several genes of the innate immune system. Single nucleotide polymorphisms (SNPs) in the Pentraxin 3 (PTX3) gene decrease antifungal clearance and phagocytosis by neutrophils and therefore increase the susceptibility to invasive mold infections. These PTX3 SNPs have been linked to an increased fungal infection risk in each of the 3 patient groups at highest risk for invasive mould infections: solid organ transplant recipients, allogeneic stem cell transplant recipients and patients with acute leukemia [7-9]. Future studies should look at the role of PTX3 in IAPA.

Apart from genetic factors, environmental factors are likely to play a role as well, as IAPA is often diagnosed in the first days after and even on the day of ICU admission. This suggests that the infection is caused by *Aspergillus* spores inhaled by the patient preceding hospital admission. Therefore, it is likely that differences in *Aspergillus* spore counts in the air (e.g. rural rather than urban, dry versus wet climate) will influence the risk of IAPA. Apart from diagnostic and genetic factors, the way health-care is organized locally may also influence the incidence of IAPA across countries and continents. Indeed, so far data on IAPA come almost entirely from tertiary care ICU centers. But even within these tertiary care ICU populations, the specific patient referral policy is likely to influence the IAPA risk. For instance, if extra-corporal membrane oxygenation is only performed at the sites included in a specific study, the patients admitted at these ICUs will often be referred from first care hospitals after conventional ventilatory support has been shown to be insufficient. These differences in ventilatory failure may not be reflected in APACHE scores. Also, patients admitted to the ICU in tertiary care centers may more often have specific underlying disease in which tertiary care hospitals are more often specialized (e.g. vasculitis, solid organ transplantation, autoimmune diseases). Finally, we have more speculative explanations for the observed differences in IAPA. Differences in influenza vaccination policies will influence the uptake of influenza vaccination and could change the severity of illness of an influenza infection in the population under study. Even more speculative is that the reported higher incidence of IAPA in recent years might be the caused by the widespread use of neuraminidase

inhibitors in patients infected with influenza. Fundamental research indicates that neuraminidase seems to play a role in the host immunity against *Aspergillus species* and blocking neuraminidase could increase the risk for *Aspergillus* superinfection [10]. Finally, the reported incidences of IAPA in ICU patients may only reflect the tip of iceberg. Some patients in the study by Schwartz *et al*. survived without treatment while others died despite best antifungal therapy. It might be that *Aspergillus* superinfection is quite common during influenza but only clinically relevant in patients admitted to the ICU.

But what is the clinical relevance of IAPA? Is it just an innocent bystander or is it truly one of the steps on the path from influenza infection to the death of these patients in the ICU? Half of the patients with IAPA in the cohort study from Dr. Schwartz *et al*. died. This is in line with the reported mortality of IAPA cases in the DB-MSG study. To try to answer the question whether the significantly higher mortality observed in patients with IAPA can be attributed to the *Aspergillus* superinfection or if it is just a marker of overall disease severity, we performed a mortality analysis on the DB-MSG study cohort. Remember, in this study 432 patients admitted to the ICU with influenza were included of whom 117 were immunocompromised according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group EORTC/MSG criteria [11]. 83 of the 432 patients (19%) were diagnosed with IAPA, and the 90-day mortality was 51%, which was substantially higher than the mortality in the 349 patients without IAPA (28%, P<0.001). A Kaplan Meier survival curve was made for patients with and without IAPA (figure 1A) and a cox regression analysis was performed to determine whether IAPA, as a time-dependent covariate, was independently associated with 90-day mortality, using the independent covariates as depicted in figure 1B [12]. In the immunocompromised subgroup, one third of patients (38 (32%)) developed IAPA and 71% died. The cox regression analysis showed that the emergence of IAPA was independently associated with 90-day mortality (adjusted Hazard Ratio (aHR) 1.944, 95% CI 1.307-.2·891, p=0.001, figure 1B) as were age (aHR 1.032, 95% CI 1.018-1·046), APACHE II (aHR 1.046, 95% CI 1.023-1.069), diabetes (aHR 1.599, 95% CI 1.092-2.342), being immunocompromised according to EORTC/MSG criteria (excluding corticosteroid use) (aHR 1.670, 95% CI 1.146-2.434) and corticosteroid therapy before ICU admission (aHR 1.118, 95% CI 1.035-1.207 per 0.1 mg/kg/day prednisone equivalent). These results strongly suggest that IAPA is independently associated with mortality in patients admitted to the ICU with influenza. Although, we acknowledge that observational data can never prove a causal relationship with 100% certainty, the association of IAPA and mortality was independent of confounders like severity of illness and being immunocompromised at ICU admission. This finding, again, confirms the relevance of diagnosing IAPA in the ICU. In accordance with recent literature, corticosteroids exposition before ICU admission

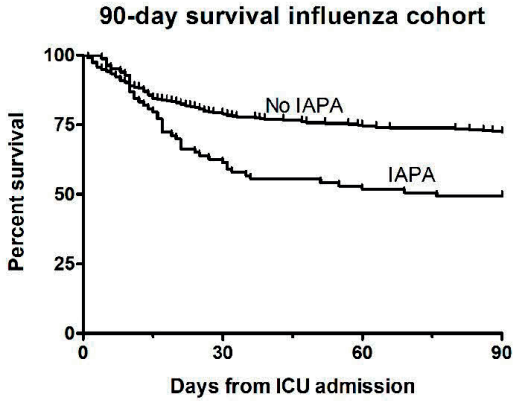

Figure 1A: Kaplan Meier 90-day survival function of the influenza cohort.
IAPA= Influenza-associated aspergillosis. ICU=Intensive Care Unit.

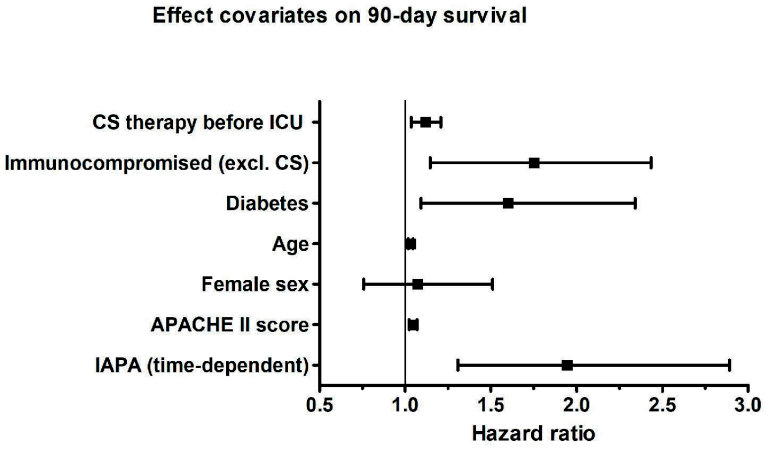

Figure 1B: Forest plot Cox regression analysis
IAPA=Influenza-associated aspergillosis. CS=Corticosteroids. CS therapy before ICU=CS therapy in the 4 weeks preceding ICU admission Immunocompromised=patients with a host factor as defined by the EORTC/MSG criteria[12].

in patients with severe influenza 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 [13, 14].

Many outstanding questions remain to be resolved. To answer these questions, the quality of future research on IAPA needs to be improved further. For this we will need a consensus definition of IAPA to be used in future studies. Therefore, a group of experts in the field of invasive fungal infections and intensive care medicine met to discuss and eventually formulate a workable set of definitions. We expect these to become publicly available as early as 2020. Future studies should try to find risk factors for IAPA, other than those already found (i.e. being immunocompromised and a higher

APACHE II score). This will allow for stratification of patients included in studies on the prevention of IAPA (e.g. with systemic or inhaled antifungal prophylaxis). It will also help the clinician when a decision needs to be made upon the invasiveness of diagnostic procedures to be done. Indeed, in a patient at very high risk for IAPA, a more invasive diagnostic strategy is justified. Once the diagnosis is made, the optimal therapy of IAPA needs to be found. Until new data arise, it is logical to treat these patients according to guidelines on the treatment of invasive aspergillosis. However, patients with *Aspergillus* tracheobronchitis may need to be treated differently. Also, it may well be that at least a subset of patients with IAPA can be treated for just a few weeks rather than a typical duration of at least 6 weeks and often many months in patients with a probable invasive aspergillosis according to the EORTC/MSG definition. Finally, we think that a better understanding of the underlying immunological mechanism and pathogenesis of IAPA is clearly needed because this may eventually lead to targeted prevention or therapy.

In conclusion, IAPA is a frequent and potentially lethal complication of influenza in critically-ill patients. While its incidence may vary between geographical regions and centers, also small primary care ICU's will see these patients if the awareness among physicians is in place. Data like the study by Schwartz *et al*. demonstrate that in patients with influenza admitted to the ICU with respiratory insufficiency a diagnostic bronchoscopy should be done to look for tracheobronchitis with biopsy of visible lesions if possible but also to sample BAL fluid. If the patient is not yet intubated, a very experienced bronchoscopist is often still able to perform a "mini-BAL" in just a few minutes while the patient is receiving high-flow nasal oxygen therapy. GM testing should be done on serum and preferentially also on BAL fluid. At ICU admission, a fungal culture on sputum or tracheal aspirates should be done. If IAPA is excluded on admission but progressive radiological and or clinical deterioration is observed during or after ICU admission, a repeated radiological and/or bronchoscopic evaluation is needed to rule out IAPA (again).

Declaration of interests

BJAR received research grants from Gilead and MSD outside the context of this study. He also received travel grants from MSD, Gilead, BMS, Jansen-Cilag, ViiV and Abbvie and received personal fees from Gilead, Viiv and Great-Lake pharmaceuticals. He served as an advisor to Gilead, ViiV, BMS, Abbvie, Jansen-Cilag and MSD. AFADS received travel grants to attend international conferences from Gilead, Pfizer and Roche outside the context of this study. JW received research grants from Pfizer and MSD outside the context of this study. He also received travel grants from MSD, Gilead and Pfizer.

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Chapter 11

Azole-echinocandin combination therapy for invasive aspergillosis. A randomized pragmatic superiority trial (DUET)

My mama always said, life is like a box of chocolates.
You never know what you're gonna get. (Forrest Gump)

RESEARCH PROTOCOL

Studying the relevance of antifungal combination therapy for invasive aspergillosis. (IA-DUET)

Short title	**IA-DUET study / HOVON 502**
EudraCT number	2020-000627-40
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Collaborating party	HOVON
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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)
A. fumigatus	Aspergillus fumigatus
A. species	Aspergillus species
AE	Adverse Event
AML	Acute Myeloid Leukemia
AR	Adverse Reaction
BAL	Bronchoscopic Alveolar Lavage
BID	Twice daily
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CRF	Case Report Form
CT	Cycle Treshold
CV	Curriculum Vitae
D	Day
DSMB	Data Safety Monitoring Board
DDI	Drug-drug interaction
EORTC	European Organisation for Research and Treatment of Cancer
EORTC/MSG	European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EU	European Union
EudraCT	European drug regulatory affairs Clinical Trials
GCP	Good Clinical Practice
GVHD	Graft Versus Host Disease
HSCT	Hematopoietic stem cell transplantation
IA	Invasive Aspergillosis
IB	Investigator's Brochure
IC	Informed Consent
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
iMTA	institute for Medical Technology Assessment
IRB	Institutional review board
IV	Intravenous
IMP	Investigational Medicinal Product
IMPD	Investigational Medicinal Product Dossier
METC	Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)
MITT	Modified intention to treat
MSG	Mycoses Study Group
PCR	Polymerase Chain Reaction
PO	Per os (oral intake)
QALY	Quality-adjusted life year
RAMS	Azole reistance associated mutations
RT	Resistance

(S)AE	(Serious) Adverse Event
SOC SOT SPC	Standard of care Solid Organ Transplant Summary of Product Characteristics
Sponsor	The sponsor is the party that commissions the organization or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor but referred to as a subsidizing party.
SUSAR SWAB	Suspected Unexpected Serious Adverse Reaction Stichting Werkgroep Antibioticabeleid (in Dutch)
TR	Tandem Repeat
Wbp	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale

Patients with underlying haematological malignancies or immunocompromised for various other reasons, are prone to fungal infections. Invasive aspergillosis (IA) is a common complication during remission inducing chemotherapy for acute leukemia or other hematological malignancies, as well as those who have undergone allogeneic hematopoietic stem cell transplantation (HSCT) or solid organ transplantation (SOT). 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% (Herbrecht et al., 2002[1]). Therefore, a randomized controlled trial assed the efficacy of voriconazole with or without anidulafungin for the treatment of IA in haematology patients to prove that combination therapy can improve outcome.[2] Among the 277 patients with IA in this study, the 6-week mortality with combination therapy was 30% lower (19.3%) than with monotherapy (27.5%), p=0.087. 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). Though, this study did not result in conclusive evidence in favor of combination therapy, it is a credible study which adds to the already existing in vitro and animal studies in support of echinocandin triazole combination therapy for IA and thus paves the way for a second larger and pragmatic clinical trial. Another important and new consideration about the management of IA is the upcoming of infections with triazole-resistant A.*fumigatus*. This is increasingly becoming a worldwide problem and leads to longer hospital stay, higher costs and is associated with a very high mortality. It is very likely that the exces-

sive use of antifungals of the triazole class in agriculture has formed the basis of this problem. Since 2018 the Dutch Working Party on Antibiotic therapy (SWAB) guideline on the management of invasive fungal infections therefore recommends upfront combination therapy (azole plus echinocandins or liposomal-amfotericine B) until resistance can be excluded as one of the treatment options for IA.

Given the evidence in favor 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 urgently needed.

Objectives

Primary objective

1. Evaluate if the survival in patients with a triazole susceptible IA can be improved when the initial therapy consists of triazole and echinocandin combination therapy instead of triazole monotherapy. This objective is captured in the primary endpoint as well as secondary endpoints 1 to 6)

Secondary objectives

1. Evaluate if a triazole/echinocandin combination therapy improves the overall quality of life and if it is a cost-effective intervention (these objectives are captured in secondary endpoint 11 and 12)
2. Evaluate the outcome of patients in which a triazole-resistant *A. fumigatus* is detected in relation to the initial antifungal therapy they had received (i.e. triazole monotherapy or combination therapy). This objective translates into secondary endpoint 7 an 8.
3. Evaluate the outcome of patients in which resistance testing is unsuccessful in function of the antifungal therapy they received. This translates into secondary endpoint 10.
4. Evaluate if the baseline serum galactomannan value and the serum galactomannan kinetics are predictive of overall 6-week survival. This translates into secondary endpoint 3 and 9.

Study design and intervention: 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 survival, 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 has been documented but when a treatment response is observed before day 28, the echinocandin can be discontinued as from day 7.

Study population

Immunocompromised patients who fulfill the EORTC/MSG host factor and mycological criteria of invasive aspergillosis ICU patients with influenza who fulfill a definition of IA specific for this population

Primary endpoint

Primary endpoint

Overall survival 42 days after the start of antifungal therapy in the MITT population

Secondary endpoints

1. Overall aspergillus attributable mortality 12 weeks after the start of antifungal therapy.
2. Overall survival 12 weeks after the start of antifungal therapy in the MITT population
3. Overall survival 6 weeks after the start of therapy in the subgroup of patients in the MITT population with a positive serum galactomannan test at baseline.
4. Overall survival 6 weeks after the start of therapy in the subgroup of non-ICU patients who fulfill the EORTC/MSG probable or proven definition (MITT population).
5. Overall survival 6 weeks after the start of therapy in the subgroup of non-ICU patients with an underlying haematological disease (MITT population)
6. Overall survival 6 weeks after the start of therapy in the subgroup of non-ICU patients without an underlying haematological disease (MITT population)
7. Overall survival 6 weeks after the start of therapy in patients that started with triazole monotherapy and in which triazole resistance is detected during follow-up (MITT population)
8. Overall survival 6 weeks after the start of therapy in patients that started with triazole-anidulafungin combination therapy and in which triazole resistance is detected during follow-up (MITT population)
9. In the subgroup of patients with a positive serum galactomannan; Kinetics of serum galactomannan levels with combination versus monotherapy
10. Outcome of patients in which resistance testing was unsuccessful
11. Time to hospital discharge (in the MITT subgroup of patients admitted to the hospital at baseline)
12. Cost-effectivity of azole-anidulafungin combination therapy

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: The safety of this combination therapy has previously been demonstrated in a large randomization clinical trial (Marr et al., 2015).[2] As a result of the underlying disease as well as the chemotherapy, serious adverse events are very frequently observed in this patient population (e.g. bleeding, life threatening infections, death due to progression of the underlying disease). The study will comprise of 4 study visits and as most patients will be hospitalized at the start of therapy few of these will be additional hospital visits on top of the standard of care.

This video presentation describes the study in more detail as well
https://www.youtube.com/watch?v=Knq58Zar4hY

1. INTRODUCTION AND RATIONALE

Invasive aspergillosis (IA) is a common complication during remission inducing chemotherapy for acute leukemia as well as in those who have undergone allogeneic hematopoietic stem cell transplantation (HSCT) or solid organ transplantation (SOT). 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% (Herbrecht et al., 2002[1]). Voriconazole blocks the synthesis of ergosterol, a part of the fungal membrane, while antifungals from the echinocandin class block the synthesis of ß-(1,3)-D glucan, a component of the cell wall. Both drugs may therefore work synergistically as suggested by in vitro studies, neutropenic animal models of IA and case series (Philip et al., 2005[3]; Petraitis et al., 2003[4]). This synergistic effect was the hypothesis of a randomized trial that assessed the efficacy of voriconazole with or without anidulafungin for the treatment of IA in haematology patients (Marr et al., 2012[2]). Among the 277 patients with IA in this study, the 6-week mortality with combination therapy was 30% lower (19.3%) than with monotherapy (27.5%), p=0.087. 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). These results were clearly promising and although we agree that in real-life in haematology patients a diagnosis of IA is indeed very often based on the combination of a positive galactomannan and pulmonary abnormalities, formal conclusions on the value of combination therapy cannot be based on a post-hoc analysis from a single clinical trial. This is the reason why, despite the 30% relative reduction in mortality that was observed, combination therapy has not been included as preferred

first choice therapy for all patients with IA in the 2017 ESCMID guideline nor in the 2017 Dutch SWAB guideline. Therefore, a second clinical trial is needed to confirm this finding.

Another important reason to study upfront combination therapy for patients with IA in the Netherlands and Belgium is the increasing incidence of triazole-resistant *A. fumigatus*. Indeed, 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 2017. Unfortunately, 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% (Vermeulen et al., 2015[5]; Montesinos et al., 2017[6]). It has also clearly been demonstrated that the overall mortality becomes very high (50-88%) when patients infected with a triazole-resistant *A. fumigatus* initially receive inappropriate voriconazole therapy and therapy is only changed at a time when it has become clinically obvious that the IA is progressing (Lestrade et al., 2018[7], van der Linden et al., 2015[8]; Chong et al., 2015[9]). These important observations recently led to a change in the treatment recommendations of the 2017 Dutch SWAB guideline on fungal infections. In the absence of any evidence from prospective studies on the treatment of IA in regions with a high prevalence of azole resistance, this guideline recommends 2 possible strategies. The first is upfront combination therapy (triazole combined with an echinocandin or liposomal amphotericin B) until resistance can be excluded. The second option, which should only be considered in non-critically ill patients and in centers that are able to perform real-time PCR as well as cultured based resistance testing on BAL samples, is to start with voriconazole monotherapy while waiting for the resistance test (Kullberg et al., 2018[10]). Unfortunately, resistance testing will not lead to an interpretable result in approximately 35% of the patients with IA. Indeed, fungal cultures remain negative in the majority of the patients with IA and PCR testing for CYP51 resistance associated mutations is not always successful either. For this subgroup of patients, the SWAB guideline recommends switching from triazole monotherapy to combination therapy as soon as it becomes clear that no resistance result will become available. The latter recommendation has been criticized when it relates to patients that are not very sick and have an infection that is limited to the lungs and that is not widespread. Indeed, some clinicians argue that close monitoring for treatment progression is a valid option as well because the poor outcome of azole resistant IA has not (yet) been convincingly demonstrated for culture negative cases.

The goals of this study are therefore 3-fold.

First, the main study and the primary endpoint will evaluate if the overall mortality can be decreased with initial azole-echinocandin combination therapy compared with triazole monotherapy in patients with IA and documented *voriconazole susceptibility.*

Second, the study design described below will also allow to study several other subpopulations; Indeed, the outcome of the following subgroups will be evaluated as well; *a*. Patients starting azole monotherapy but who switch to directed therapy when it has become clear that the infection is caused by an azole resistant A. fumigatus. *b*. patients in which eventually no resistance data become available in relation to the treatment they received.

Third, we want to evaluate what the outcome is of patients that turn out to be infected with a triazole resistant A. fumigatus who started with a triazole-echinocandin combination therapy.

Please note that a 20-minute presentation with illustrated slides has been put together in order to explain the background and the design of the study. This presentation is available via this URL: https://www.youtube.com/watch?v=Knq58Zar4hY and may help to understand the design and logistics of the study as an introduction to the full protocol

2. OBJECTIVES

Primary objective

1. Evaluate if the survival in patients with a triazole susceptible IA can be improved when the initial therapy consists of triazole and echinocandin combination therapy instead of triazole monotherapy. This objective is captured in the primary endpoint as well as secondary endpoints 1 to 6)

Secondary objectives

1. Evaluate if a triazole/echinocandin combination therapy improves the overall quality of life and if it is a cost-effective intervention (these objectives are captured in secondary endpoint 11 and 12)
2. Evaluate the outcome of patients in which a triazole-resistant *A. fumigatus* is detected in relation to the initial antifungal therapy they had received (i.e. triazole monotherapy or combination therapy). This objective translates into secondary endpoint 7 an 8.
3. Evaluate the outcome of patients in which resistance testing is unsuccessful in function of the antifungal therapy they received. This translates into secondary endpoint 10.
4. Evaluate if the baseline serum galactomannan value and the serum galactomannan kinetics are predictive of overall 6-week survival. This translates into secondary endpoint 3 and 9.

3. STUDY DESIGN

A **non-blinded phase 3 multicenter randomized pragmatic clinical trial**.
Intervention: Add anidulafungin IV to the standard of care therapy that consists of a triazole (voriconazole or isavuconazole or posaconazole).

In both the intervention and control group, the triazole will be given for at least 6 weeks. In the intervention group the echinocandin will be discontinued after 28 days but when a treatment response is observed and the patients can be discharged from the hospital, the echinocandin can be discontinued from day 7 onwards according to the choice of the treating physician. Figure 1 illustrates the study design and the patient flow in the study. The youtube presentation https://www.youtube.com/watch?v=Knq58Zar4hY also clearly explains this flowdiagram.

4. STUDY POPULATION

4.1. Population (base)

The study population will consist of patients age 18 or older that fulfill the host-factor definition of the EORTC/MSG[11] in combination with any pulmonary infiltrate and a positive fungal culture from a bronchoalveolar lavage or positive serum (optical density ≥0.5) or BAL galactomannan (optical density ≥1.0). This not only includes patients with proven or probable IA but also patients with a pulmonary infiltrate that does not comply with the EORTC radiological criteria (halo, nodule, cavitary lesion). We consider the inclusion of this latter patient population important as well because not only the mortality of these patients is comparable to patients with an EORTC/MSG probable IA but clinicians also uniformly treat these patients in the same way as they treat patients with probable IA (Nucci et al., 2010[12]).

The host-factor definition of the EORTC/MSG implies that not only patients with an underlying haematological disease can be included but any patients that is sufficiently immunocompromised to fulfil the host-factor definition. Furthermore, ICU patients with a predicted mortality not exceeding 50% and admitted with influenza and respiratory insufficiency can be included as well as this has recently been described as an important risk factor for IA by Schauwvlieghe et al[13].

4.2. Inclusion criteria

Patients should fulfill the following inclusion criteria:

1. 18 years or older

2. Have started or will start voriconazole or isavuconazole (or posaconazole if voriconazole or isavuconazole cannot be given as per treating physician's decision) as antifungal therapy on the baseline visit.
3. For all patients: presence of one of the EORTC/MSG host factors as defined in appendix 1 or being admitted to the ICU with influenza
4. For non-ICU patients or ICU patients without influenza: Meet the EORTC/MSG clinical criterium (appendix 1)
5. For non-ICU patients or ICU patients without influenza: Meet the mycological criterium (appendix 1) or fulfil inclusion criterium 7
6. For ICU patients with influenza we consider an isolated positive sputum culture for Aspergillus spp. insufficient as a mycological criterium. Therefore, in these patients only one of the following mycological criteria are acceptable; Serum galactomannan □0.5, BAL galactomannan □1.0 or *Aspergillus* spp. cultured in BAL fluid.
7. Please note that patients with AML receiving chemotherapy or patients with ALL receiving or having received corticosteroid therapy within the last 4 weeks in the context of their pre-phase, induction, consolidation, intensification or interphase treatment as well as patients receiving systemic immunosuppressive therapy for GVHD can be included before the mycological criterium is fulfilled on condition that they fulfill the EORTC/MSG lung CT radiology criteria (halo sign, well-described nodule, cavity as described in appendix 1) at the time of inclusion and as long as the mycological test results are expected to become available within 96 and no later than 7 days after inclusion. If these test results turn out to be negative, the patient will be withdrawn from the study and further treatment is at physician's discretion.

4.3. Exclusion criteria

1. Known history of allergy, hypersensitivity or serious reaction to azole or echinocandin antifungals;
2. Patients with chronic invasive aspergillosis or a chronic non-invasive aspergillus infection (e.g. aspergilloma) defined as the clinical or radiological sign of infection being present for >28 days.
3. Receipt of itraconazole, voriconazole, posaconazole or isavuconazole as prophylaxis for at least 7 days in the 14 days preceding the date of the first radiological signs of the *Aspergillus* infection. Patients in which the most recent serum level of the triazole given as prophylaxis was subtherapeutic can be included [(*)].
4. Receipt of echinocandin prophylaxis for >96 hours in the preceding 7 days
5. Receipt of systemic antifungal treatment with an echinocandin or an azole for the current episode of invasive aspergillosis for a duration of > 96 hours.

6. For patients in the Netherlands only: Diagnostic testing to exclude azole resistance will not be possible (sputum cultures are negative and BAL sampling will not be performed)
7. ICU patients only: Patients with a sequential organ failure assessment (SOFA) score >11 at the time of screening for the study are excluded. If randomization is done >24 hours after screening the calculation should be repeated before the patient can be randomized (appendix 3)
8. ICU patients only: Patients in which weaning from the ventilator or ECMO system is deemed unlikely due to irreversible lung damage
9. Patients with any condition which, in the opinion of the investigator, could affect patient safety, preclude evaluation of response (e.g. because survival beyond 6 weeks is unlikely due to the underlying disease status)
10. Patient previously included in this study

(*) Subtherapeutic levels are defined as itraconazole (parent compound only) <0.5 mg/L or posaconazole <0.7mg/L or voriconazole <1.0mg/L or isavuconazole <1.0mg/L

5. TREATMENT OF SUBJECTS

5.1. Investigational product/treatment

Anidulafungin

Detailed information on anidulafungin, the investigational product used in this study can be found in the SPC. A short summary is given here. The information currently available on combination therapy with a triazole and an echinocandin for the treatment of IA is described below (6.2).

Mechanism of action

Anidulafungin is a semi-synthetic echinocandin, a lipopeptide synthesised from a fermentation product of Aspergillus nidulans. Anidulafungin selectively inhibits 1,3-ß-D glucan synthase, an enzyme present in fungal, but not mammalian cells. This results in inhibition of the formation of 1,3-beta-D-glucan, an essential component of the fungal cell wall. Anidulafungin has shown fungicidal activity against Candida species and activity against regions of active cell growth of the hyphae of Aspergillus fumigatus.

Chemical Name

Anidulafungin has the chemical name 1-[(4R,5R)-4,5- Dihydroxy-N2- [[4”-(pentyloxy) [1,1’:4’,1”-terphenyl]- 4- yl]carbonyl]- Lornithine]echinocandin B.

Name of medicinal product

ECALTA 100 mg powder for concentrate for solution for infusion

Method of administration

For intravenous use only.

ECALTA should be reconstituted with water for injections to a concentration of 3.33 mg/mL and subsequently diluted to a concentration of 0.77 mg/mL.

Dosage & duration

Adult patients receive a loading dose on day-1 of 200mg IV, followed by 100 mg daily thereafter.

Anidulafungin will be given for at least 7 days but no longer than 28 days. Please note that this refers to the MITT primary endpoint population. In patients in which triazole resistance is demonstrated or in which the results of the resistance test are inconclusive, the treatment duration of combination therapy can be much longer. These patients are not included in the MITT primary endpoint population.

Intervention

Treatment with a triazole (voriconazole or isavuconazole or posaconazole) + anidulafungin IV. The triazole is administered for at least 6 weeks while anidulafungin is given for at least 7 and a maximum of 28 days.

Comparator

Treatment with a triazole (voriconazole or isavuconazole or posaconazole) for at least 6 weeks.

In both groups, the route of administration is according to the choice of the treating physician as well as the decision to perform or not perform therapeutic drug monitoring of the triazole drug.

5.2. Use of co-intervention (if applicable)

Not applicable

5.3. Resistance testing

At the end of 2017, the Dutch SWAB guideline on the treatment of invasive fungal infections was updated. To take the increasing incidence of triazole resistance in the Netherlands into account, this guideline describes 2 preferred treatment strategies for patients with invasive aspergillosis. One strategy is to start with combination therapy (azole in combination with echinocandin or azole with liposomal amphotericin-B) until resistance test results become available. This is an option for critically ill patients or in

a setting where real-time resistance testing is not readily available. In a setting where real-time and state-of-the-art resistance testing is available, treatment with triazole monotherapy is recommended as an alternative strategy.

To allow for the treatment with triazole monotherapy in the Dutch patient population of this study, resistance testing will be done in all patients included in the Netherlands at the study site or at Erasmus MC if not part of the standard local diagnostic work-up. Furthermore, all Belgian sites in which triazole resistance testing is not the current standard of care, will be given the opportunity to send BAL samples to a central lab in Belgium (UZ Leuven or AZ Sint-Jan in Brugge) for real-time resistance testing free of charge. All participating centers will also receive the lab tools to perform in-house phenotypic resistance testing using VIPcheck™ free of charge (see below).

Resistance testing will be performed using a combination of phenotypic as well as genotypic resistance tests. *Phenotypic resistance testing* means that the fungus is cultured in the presence and absence of triazole drugs to observe suppression of growth in the presence of triazole drugs. It is currently certainly not the standard of care diagnostic procedure in all Belgian centers. Centers where this is not a standard procedure will receive the necessary tools to implement phenotypic resistance testing with the use of the VIPcheck™. This test was developed and validated by Radboud UMC.[6,14] The VIPcheck™ is a test consisting out of a 4-well plate in which three of the four wells contain agar supplemented with an azole (voriconazole, itraconazole and posaconazole) and the fourth functions as a growth control. If an *Aspergillus* strain grows in a well with an azole, it is very likely that this strain is azole-resistant. This test will be performed locally at the sites where this is the standard of care and the presence of resistance will be confirmed with the European Committee on Antibiotic Susceptibility Testing (EUCAST) method at the reference mycology lab of the Netherlands or Belgium (Radboud UMC and UZ Gasthuisberg Leuven). Sites that are currently not using the VIPcheck but are willing to use it can contact the study team to get the test sent to them. *Genotypic resistance testing* means that a PCR test is used to document the absence or presence of certain mutations in the DNA of the fungus that are known to result in phenotypic resistance. For this purpose, 1.5 to 2ml of the BAL sample of each patient will be submitted to the central lab to test for the presence of with the commercially available AsperGenius®. This PCR allows for the simultaneous detection of *Aspergillus* species and identification of the most common mutations circulating in Belgium and the Netherlands (TR_{34}/L98H or TR_{46}/T289A/Y121F) in the *A. fumigatus Cyp51A* gen by using melting curve analysis.

With this state-of-the-art diagnostic approach, it is safe to start with azole monotherapy for the treatment of invasive aspergillosis and the treating physician will not be tempted to change therapy from azole monotherapy to combination therapy because of fear for resistance.

5.4. Escape medication (if applicable)

Not applicable

6. INVESTIGATIONAL PRODUCTS

See also 5.1 and the SPC for more details on anidulafungin, the IP used in this study. Here we describe the findings from non-clinical and clinical studies on combination therapy with a triazole and an echinocandin as combination therapy for the treatment of IA.

6.1. Summary of findings from non-clinical studies concerning triazoles and echinocandins as combination therapy

Findings from non-clinical studies concerning the investigational products show a positive effect of combination therapy in the treatment of IA. A study by Kirkpatrick et al.[15] in which the efficacy of caspofungin and voriconazole combination therapy was evaluated in an immunocompromized guinea pig model of IA, showed a reduced mortality in the combination group compared to the single therapy dosage. In the combination therapy the colony counts were reduced compared to those obtained with either Amfo B alone or voriconazole alone. Only combination therapy resulted in more sterile cultures of organs 96 h after completion of therapy than those achieved with the other therapeutic regimens examined in these experiments. The authors concluded that the combination therapy has relevant clinical importance and the need for further clinical studies to investigate the use of combination therapy for invasive aspergillosis. In a neutropenic rabbit model of invasive pulmonary aspergillosis, the combination of voriconazole and anidulafungin was superior to single agent therapy with respect to mean pulmonary fungal burden and survival, among other measures.[16]

6.2. Summary of findings from clinical studies regarding triazoles and echinocandins as combination therapy.

A non-randomized observational study by Singh et al., 2006[17], in which the efficacy of combination of voriconazole and caspofungin as primary therapy for invasive aspergillosis in solid organ transplant recipients was assessed, showed that the survival at 90 days was 67,5% (27/40) in the combination group compared to 51% (24/47) in the L-AmB group, a difference that was not statistically significant. In transplants recipients with renal failure and in those with A. fumigatus infection, combination therapy was independently associated with an improved 90-day survival in multivariate analysis. This study has important limitations as the control group did not receive triazole monotherapy but rather liposomal amphotericin-B as they were treated in the years 1999

and 2002 when voriconazole was not yet available. The only randomized clinical trial in which triazole monotherapy was compared with triazole-echinocandin combination therapy is the study by Marr et al., 2015, in which the efficacy of voriconazole with or without anidulafungin for the treatment of IA in haematology patients was assessed.[2] Patients were 16 years or older, had an underlying hematologic malignancy and/or had undergone a hematopoietic cell transplantation and were diagnosed with probable or proven IA according to the EORTC/MSG criteria. Patients received either voriconazole and a placebo or voriconazole in combination with anidulafungin. The primary end point was all-cause mortality at 6 weeks after inclusion and one of the secondary endpoints was the 12-week overall mortality. 459 patients were enrolled and were randomly assigned to one of the treatment arms. The miTT population included 277 patients who had confirmed proven or probable IA by the end of the first study week; 135 patients received combination treatment and 142 received monotherapy. The median duration of combination treatment was 14 days (range, 1 to 29); the median duration of voriconazole treatment was 42 days (range, 1 to 48 days). The mortality at 6 weeks in the miTT population was 19.5% (26 of 135) for combination treatment and 27,8% (39 of 142) for monotherapy (with a difference of -8.2%; 95% CI, -,19.0 to 1.5; 2-sided P= 0.087). The mortality at 12 weeks was 29.3% (39 of 135) for the combination treatment and 39.4% (55 of 142) for monotherapy (difference -10.1%; P=0.077). A post-hoc analysis of mortality in the patients with confirmed diagnosis of probable IA that was based on radiographic findings and galactomannan antigen positivity in serum or BAL was performed. All-cause mortality was 15,7% (17 of 108) in the combination therapy group compared with 27,3% (30 of 110) in the monotherapy group (p=0.037). These results were clearly promising and we agree that in real-life a diagnosis of IA in hematology patients is indeed very often based on the combination of a positive galactomannan and pulmonary abnormalities. However, formal conclusions on the value of combination therapy cannot be based on a post-hoc analysis from a single clinical trial. The authors concluded that though results do not provide a conclusive evidence of superiority they add to the support of combination therapy for IA.

6.3. Summary of findings from clinical studies regarding triazole monotherapy

Voriconazole, available as an oral and IV formulation has been the first-line standard of care therapy for patients with invasive aspergillosis since 2003. Indeed, in a pivotal randomized clinical trial in which voriconazole was compared with IV amphotericin-B deoxycholate, the overall 12-week survival was significantly higher in patients treated with voriconazole (71% versus58%).[1]

Isavuconazole, available in capsule and IV formulation, is another triazole registered for the treatment of IA. In the large randomized SECURE trial, 527 patients with inva-

sive mold infection were randomized to receive either IV isavuconazole followed by IV or oral (PO) isavuconazole versus IV voriconazole followed by IV or PO voriconazole. The majority of patients in both groups had underlying hematologic malignancy (82% in the isavuconazole group versus 86% in the voriconazole group). The primary endpoint was all-cause mortality at 6 weeks. At 6 weeks, 19% of patients in the isavuconazole group died compared to 20% in the voriconazole group, a difference that did not meet statistical significance. Drug-related adverse events were significantly higher in the voriconazole group compared to the isavuconazole group (60% versus 42%, $p < 0.001$), and permanent drug discontinuation was lower in the isavuconazole group compared to the voriconazole group (8% versus 14%).[18]

Posaconazole, available as tablets and IV formulation, is approved for the use as prophylaxis against invasive fungal infections in patients with acute myeloid leukemie and GVHD. It's in vitro activitity against *Aspergillus species* is comparable to isavuconazole. A phase III study in which posaconazole is compared with voriconazole is fully enrolled and results are expected in the near future. In patients who cannot tolerate voriconazole and/or isavuconazole, posaconazole is used off-label for the treatment of IA and this is allowed in this trial if the treating physicians things that posaconazole is the best treatment option available for the patient.

6.4. Summary of known and potential risks and benefits

Anidulafungin has an excellent safety profile with reduced toxicities, compared to other licensed antifungal agents. Adverse events that were observed in clinical trials are described in the SPC.

6.5. Description and justification of route of administration and dosage

Anidulafungin will be used at the licensed dose of a 200mg IV loading dose on day 1 and 100mg QD IV thereafter. No dose adjustment is needed in patients with renal or hepatic insufficiency of any grade.

6.6. Dosages, dosage modifications and method of administration

Triazoles

All patients will receive triazole therapy as per standard of care and this will be initiated by the treating physician before the patient is included in the study. Therefore, the triazoles (voriconazole or isavuconazole or posaconazole) are not considered study drugs.

Voriconazole or isavuconazole or posaconazole will be dosed according to the SPC and according to the route of administration (IV or orally) that is preferred by the treating physician. However, the dose may be changed based on therapeutic drug monitoring levels according to the local standard of care.

Anidulafungin:
Anidulafungin (Ecalta) is available as an intravenous formulation only. It will be used at the licensed dose of a 200mg loading dose on day 1 and 100mg QD thereafter. No dose adjustment is needed in patients with renal or hepatic insufficiency of any grade.

6.7. Preparation and labeling of Investigational Medicinal Product

The investigational product will be labeled at the Erasmus MC trial pharmacy according to the relevant good manufacturing practice (GMP) guidelines and with the use of a study label compliant with the Annex 13 EU directive. The investigational products will be shipped to the study sites after labeling and resupplies can be ordered at Erasmus MC.

6.8. Drug accountability

The pharmacy at the study site will carry out the drug accountability. Batch numbers of medication administered to the patients will be recorded in the patient files and CRF. The central pharmacy at Erasmus MC or at the local site will be responsible for the destruction of medication that is returned pursuant to the ICH/GCP Guidelines, local regulations and the investigator's institutional policies. Clinical supplies will be received by a designated person at the study site, handled and stored safely and properly, and kept in a secured location to which only the investigator, a pharmacist or its designated assistant have access. Clinical supplies are dispensed in accordance with the protocol. The investigator is responsible for keeping accurate records of the clinical supplies, the amount dispensed to the ward and returned to the pharmacy by the ward as well as the disposition at the end of the study. The investigator can delegate this task to the hospital pharmacist. A stock of at least 25 vials of 100mg should be in place at the site as long as the study is open for inclusion. If the stock falls below 25 vials, the investigator or the hospital pharmacist will order a new stock of 25 vials, using the order form that will be provided by the Erasmus MC trial pharmacy unit at the time of the first delivery. Ordering more vials is possible after sending a request to the study team (duet.study@erasmusmc.nl)

7. METHODS

Primary hypothesis

For the treatment of IA, combination therapy of voriconazole or isavuconazole or posaconazole with anidulafungin will improve the overall survival compared with voriconazole or isavuconazole or posaconazole monotherapy.

7.1. Study parameters/endpoints

7.1.1. Main study endpoint

Overall survival 42 days after the start of antifungal therapy in the MITT population

7.1.2. Secondary study endpoints

1. Overall aspergillus attributable mortality 12 weeks after the start of antifungal therapy(*).
2. Overall survival 12 weeks after the start of antifungal therapy in the MITT population
3. Overall survival 6 weeks after the start of therapy in the subgroup of patients in the MITT population with a positive serum galactomannan test at baseline.
4. Overall survival 6 weeks after the start of therapy in the subgroup of non-ICU patients who fulfill the EORTC/MSG probable or proven definition (MITT population).
5. Overall survival 6 weeks after the start of therapy in the subgroup of non-ICU patients with an underlying haematological disease (MITT population)
6. Overall survival 6 weeks after the start of therapy in the subgroup of non-ICU patients without an underlying haematological disease (MITT population)
7. Overall survival 6 weeks after the start of therapy in patients that started with triazole monotherapy and in which triazole resistance is detected during follow-up (MITT population)
8. Overall survival 6 weeks after the start of therapy in patients that started with triazole-anidulafungin combination therapy and in which triazole resistance is detected during follow-up (MITT population)
9. In the subgroup of patients with a positive serum galactomannan; Kinetics of serum galactomannan levels with combination versus monotherapy
10. Outcome of patients in which resistance testing was unsuccessful
11. Time to hospital discharge (in the MITT subgroup of patients admitted to the hospital at baseline)
12. Cost-effectivity of azole-anidulafungin combination therapy

(*) Aspergillus attributable mortality is defined according to Vidal G et al. (with some modifications) as one of the following[22]:

When selecting one of the 4 options below, please consider the immediate cause of death as the disease process, injury, or complication immediately preceding death.

IA is considered the cause of death (=IA attributable mortality)

1. IA was considered the cause of death when the immediate cause of death was due to this infection. Examples are neurological complications of an aspergillus infection

that disseminated to the brain, lung bleeding or respiratory insufficiency in a patient with pulmonary aspergillosis *or*

2. IA was judged to have played a major role if death would not have occurred had the patient not had IA, even though another condition was present that also contributed to death. Examples are toxicity, interactions and other side effects of antifungal treatment that played a major role in the cause of death. Another example is a pseudomonas bacteremia in a patient with a cavitating pulmonary aspergillosis in which the lungs are considered the most likely source of the bacteremia.

IA contributed to the death of the patient: Mortality is not considered attributable to IA but rather contributable if IA or treatment of IA was defined as playing a minor role but probably not essential in explaining the patient's death but arguably did play some role in the event. An example is a patient an aspergillus infection as well as severe uncontrolled gastrointestinal GVHD at the time of death

IA did not contribute nor cause the death of the patient

Mortality was classified as not related to IA if there was a clear other cause of death

Unknown

If insufficient data were present about the circumstances of the death of the patient

7.1.3. Pragmatic study design

We have tried to design the study in such a way that the prompt inclusion of patients is facilitated as much as possible.

1. Registration trials of new antifungal drugs typically only include patients that fulfill the strict lung CT radiology criteria (e.g. halo sign) as described in appendix 1. However, in real-life as much as 50% of the patients treated for invasive aspergillosis do not have these typical abnormalities but are treated on the basis of the presence of a host factor, a positive mycological test and a (non-typical) pulmonary infiltrate. The outcome of these patients is similar to those with the typical pulmonary infiltrate.[12] Therefore, we consider the exclusion of these patients undesirable in the context of a pragmatic trial.
2. Typically, patients that have received antifungal therapy for more than 48-96 hours at the time of inclusion are excluded from registration trials. Again, this leads to many patients being excluded from study participation. To avoid this, we will allow that patients at high risk of IA (patents with AML or grade II or higher GVHD) are included at the time the triazole antifungal therapy is initiated by the treating physician even when the mycological criterium is not yet met. These patients will

be followed until day 7 and will leave the study if at that time the mycological tests have turned out to be negative.

3. While the previous randomized study on the use of combination antifungal therapy for IA only included patients with an underlying haematological disease, there is no reason to believe that the hypothesis of improved survival with combination therapy only applies to these patients. Therefore, our study population will not be limited to this subgroup of patients.
4. Our study design has overall mortality as the primary endpoint and no compulsory follow-up CT scans are required.
5. Given the 100% objective primary endpoint we have decided not to use a double-blind design. This will make the study logistics and therefore the inclusion of patients more straightforward. By offering state-of-the-art triazole resistance testing to all patients, the presence of triazole resistance is made very unlikely and therefore the treating physician can be reassured that the treatment given to patients in the control arm is a fully active treatment.

7.1.4. Definitions of the patient populations

ITT: All patients randomized as registered in the IVRS

MITT: All patients randomized as registered in the IVRS, in whom the positive mycological test was available at the time of randomization or became available within 8 days after randomization will be included in the MITT population *unless* resistance to voriconazole was documented by culture or PCR (VIPcheck or Aspergenius, see below). Because Dutch guidelines currently recommend initiating combination antifungal therapy if the presence of an azole resistant A. fumigatus infection cannot be excluded by PCR or culture, Dutch patients in whom the resistance PCR or culture turns out unsuccessful will be excluded from the MITT population as well. The antifungal therapy given to these patients will be decided upon by their treating physician (see also flowdiagram) .

ICU population: Patients already admitted to the ICU at the time of the start of triazole antifungal therapy

7.2. Randomisation, blinding and treatment allocation

This is a phase 3 non-blinded non-placebo controlled pragmatic trial. Randomization will be stratified according to the following 3 risk groups: Acute myeloid leukemia, graft-versus host disease and other.

Randomization and concealed allocation will be performed and guaranteed via randomization using blocks of different length and with the use of a randomization module

in the eCRF (Alea). Subjects will be randomized to a 1:1 allocation into 2 treatment group: monotherapy versus combination therapy.

7.3. Study procedures

The study procedures involve a screening visit and follow-up up to week 24 for overall mortality. Taking the pragmatic design into account, this means that a formal hospital visit is not required for the primary endpoint evaluation after the patient is discharge from the hospital. However, investigators and patients will be encouraged to facilitate the collection of additional data as described in appendix 2 as much as possible to allow for the evaluation of several secondary endpoints in a large study population. Because these data are part of routine medical registration procedures (e.g. use of blood products, hospital days, ICU days, use of medication, blood test results) this will not obstruct the analysis of these secondary endpoints.

All patients will be receiving triazole therapy as prescribed by their treating physician at the time of screening for study participation. Monitoring for treatment related adverse events will be performed as per standard of care during triazole antifungal therapy and typically includes liver enzyme monitoring at least once a week during the first weeks of therapy. Given the overall very good safety profile of anidulafungin and the fact that it can only be administered intravenously at the hospital, no additional safety evaluations are needed.

7.4. Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons. Patients leaving the study will receive treatment as deemed appropriate by their treating physician

After randomization, the following patients will leave the interventional part of the study, but clinical data will continue to be collected:

1. Patients in whom none of the mycological criteria as described in appendix 1 have become positive within 8 days after randomization. This may be as soon as 48hrs after (e.g. if serum and BAL galactomannan testing and fungal culture result is final 48hrs after randomization).
2. Patients in the Netherlands in whom the resistance tests were unsuccessful (aspergillus culture remained negative and aspergillus PCR demonstrated the presence of A. fumigatus but the resistance PCR is unsuccessful) will receive further treatment according to the choice of the treating physician because guidelines recommend to treat these patients with an azole in combination with a second antifungal drug. If the patient was randomized to the anidulafungin arm, the option will be given to

continue anidulafungin for up to 4 weeks, but these patients will be excluded from the MITT population (see 7.1.4) The 24-week outcome of these patients will be registered. In Belgium, currently no guidelines are in place recommending combination therapy for this patient group. Therefore, this does not apply to Belgian patients. Please note that patients in whom the Aspergenius PCR documents the presence of an aspergillus spp. other than fumigatus will remain in the study and will be included in the MITT population. This specific conclusion will be drawn when the aspergillus spp. PCR is positive with a CT value of 35 or lower but the aspergillus fumigatus PCR is negative as this demonstrates the presence of a non-fumigatus aspergillus infection.

7.5. Replacement of individual subjects after withdrawal

As anidulafungin has limited side-effects, a low percentage of withdrawal can be expected, and patients withdrawn from the study will not be replaced.

7.6. Follow-up of subjects withdrawn from treatment

Patients withdrawn from the study will be followed for overall mortality only

7.7. Premature termination of the study

One interim futility analysis will be performed at the time when the 6-week survival of 50% of the planned sample size of 474 evaluable patients (=237) has become available. If this interim analysis shows that the conditional power after further enrolment of the remaining study population of observing the anticipated 33% reduction in the incidence of the primary endpoint in favor of combination therapy is <10%, the study team (the PI's from Erasmus MC, UZ Leuven and Radboud UMC, the study statistician, a delegate from KCE and from ZONMW and a patient representative) will meet to decide upon further enrollment. This decision will not only take the conditional power into account but also the recruitment speed as well as the relative decrease in mortality observed with the intervention under study (e.g. it may still be useful to continue the study if a 50% rather than a 30% decrease in overall mortality is observed when the conditional power to demonstrate a 30% is <10% as a result of a lower than expected overall absolute mortality in the control arm).

8. SAFETY REPORTING

Background regarding the safety reporting paragraph of this study.

According to the current Dutch SWAB guideline, combination therapy consisting of an azole and an echinocandin (e.g. anidulafungin, the IMP in this study) is one of the

treatment options for patients with IA. Another treatment option recommended in this guideline is azole monotherapy. In Belgium, azole monotherapy is the current standard of care. Combination therapy with voriconazole and anidulafungin has been studied in a preceding phase III study. In this study, the number of AE and SAE in the combination therapy arm was comparable to the azole monotherapy arm. Therefore, in this study 2 treatment options with a well-established safety profile will be compared.

Many if not the majority of the patients in this study will be receiving intensive chemotherapy or suffer from graft-versus-host-disease and its related AEs (e.g. anemia, leuco -or thrombocytopenia, diarrhea, mucositis, nausea, vomiting, alopecia, fever, bacteremia, fatigue, headache, bacterial and viral infections infection.

The study described in this protocol was designed as a pragmatic trial of which the goal is to compare two treatment options in a setting that simulates the treatment of IA in real-life as much as possible. This will the extrapolation of the study results as straightforward as possible.

For the reasons mentioned above, we think that registering all (S)AE will not only be very time consuming because the average number of AE per patient is expected to be very high but will not increase patient safety. We therefore will not register AE but only SAE with the limitations mentioned below.

8.1. Definitions

Serious adverse event (SAE)

A serious adverse event is defined as any untoward medical occurrence or effect that at any dose:

- Results in death
- Is a life-threatening event (i.e. the patient was at immediate risk of death at the time the reaction was observed)
- Requires hospitalization or prolongation of an existing hospitalization
- Results in significant or persistent disability or incapacity
- Is a congenital anomaly or birth defect
- Is an important medical event (i.e. important adverse events that are not immediately life threatening or do not result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the above characteristics/ consequences, including suspected transmission of infectious agents by a medicinal product).

Suspected unexpected serious adverse reaction (SUSAR)

A suspected unexpected serious adverse reaction is defined as all **suspected** Adverse Reactions (AR) which occur in the trial and that are both **unexpected** and **serious**.

Suspected adverse reactions are those AEs of which a reasonable causal relationship to any dose administered of the investigational medicinal product and the event is suspected. Unexpected adverse reactions are adverse reactions, of which the nature, or severity, is not consistent with the applicable product information (e.g. Investigator's Brochure for an unapproved IMP or Summary of Product Characteristics (SPC) for an authorized medicinal product).

Overdose
This refers to the administration of a quantity of a medicinal product given per administration or cumulatively, which is above the maximum recommended dose according to the authorised product information. Clinical judgement should always be applied.

Misuse
This refers to situations where the medicinal product is intentionally and inappropriately used not in accordance with the terms of the marketing authorisation.

Abuse
This corresponds to the persistent or sporadic, intentional excessive use of a medicinal product, which is accompanied by harmful physical or psychological effects.

Occupational exposure
This refers to the exposure to a medicinal product, as a result of one's professional or non-professional occupation. It does not include the exposure to one of the ingredients during the manufacturing process before the release as finished product.

Medication error
This is an unintended failure in the drug treatment process that leads to or has the potential to lead to harm to the patient.

In accordance to section 10, subsection 1, of the WMO, the investigator will inform the subjects and the reviewing accredited METC/IRB if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the accredited METC/IRB, except insofar as suspension would jeopardize the subjects' health. The investigator will take care that all subjects are kept informed.

8.2. Adverse event

8.2.1. Reporting of adverse events

Adverse events that do not fulfill the definition of Serious adverse Event will not be reported.

8.3. Serious Adverse Events

8.3.1. Reporting of serious adverse events

Serious Adverse Events (SAEs) will be reported from the first administration of treatment according to protocol until day 14 days after the last dose of the IMP or until the start of subsequent systemic therapy for the disease under study, if earlier.

Serious adverse events (including death) occurring after day 42 days should also be reported if considered at least possibly related to the investigational medicinal product by the investigator.

SAEs must be reported to HOVON Data Center **within 3 days** after the event was known to the investigator, using the SAE report form provided. This initial report should contain a minimum amount of information regarding the event, associated treatment and patient identification, as described in the detail in the instructions for the SAE report form. Complete detailed information should be provided in a follow-up report within a further 7 business days, if necessary.

The following events do not require to be reported as a serious adverse event:

- Relapse/Progression of the disease under study. However, death or complications as a result of disease progression should be reported as serious adverse events if occurring within 14 days after last dose of study drug.
- Hospitalization for protocol therapy administration. Hospitalization or prolonged hospitalization for a complication of therapy administration will be reported as a serious adverse event.
- Hospitalization for diagnostic investigations (e.g., scans, endoscopy, sampling for laboratory tests, bone marrow sampling) that are not related to an adverse event. Hospitalization or prolonged hospitalization for a complication of such procedures remains a reportable serious adverse event.
- Prolonged hospitalization for technical, practical, or social reasons, in absence of an adverse event.
- Hospitalization for a procedure that was planned prior to study participation (i.e. prior to registration or randomization). This should be recorded in the source documents. Prolonged hospitalization for a complication of such procedures remains a reportable serious adverse event.

8.3.2. Causality assessment of serious adverse events

The investigator will decide whether the serious adverse event is related to trial medication, i.e. any of the products from the protocol treatment schedule. The decision will be recorded on the serious adverse event report. The assessment of causality is made by the investigator using the following:

Causality term	Assessment criteria*
Certain	¨ Event or laboratory test abnormality, with plausible time relationship to drug intake ¨ Cannot be explained by disease or other drugs ¨ Response to withdrawal plausible (pharmacologically, pathologically) ¨ Event definitive pharmacologically or phenomenologically (i.e. an objective and specific medical disorder or a recognised pharmacological phenomenon) ¨ Rechallenge satisfactory, if necessary
Probable /Likely	¨ Event or laboratory test abnormality, with reasonable time relationship to drug intake ¨ Unlikely to be attributed to disease or other drugs ¨ Response to withdrawal clinically reasonable ¨ Rechallenge not required
Possible	¨ Event or laboratory test abnormality, with reasonable time relationship to drug intake ¨ Could also be explained by disease or other drugs ¨ Information on drug withdrawal may be lacking or unclear
Unlikely	¨ Event or laboratory test abnormality, with a time to drug intake that makes a relationship improbable (but not impossible) ¨ Disease or other drugs provide plausible explanations
Conditional / Unclassified	¨ Event or laboratory test abnormality ¨ More data for proper assessment needed, or ¨ Additional data under examination
Unassessable / Unclassifiable	¨ Report suggesting an adverse reaction ¨ Cannot be judged because information is insufficient or contradictory ¨ Data cannot be supplemented or verified

8.3.3. Follow up of serious adverse events

All serious adverse events will be followed clinically until they are resolved or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

Follow up information on SAEs should be reported monthly until recovery or until a stable situation has been reached. The final outcome of the SAE should be reported on a final SAE report.

8.3.4. Processing of serious adverse event reports

HOVON Data Center will forward all SAE reports within 24 hours of receipt to the principal investigator.

The HDC safety desk will evaluate if the SAE qualifies as a suspected unexpected serious adverse reaction (SUSAR).

The SmPC will be used as a reference document for expectedness assessment.

Where reporting of SAEs to the ethics committee is required by national laws or regulations or by the procedures of the ethics committee, HOVON Data Center will report those SAEs by means of a six-monthly SAE line listing.

8.4. Reporting Suspected Unexpected Serious Adverse Reactions

The HDC Safety Desk, on behalf of the sponsor, will ensure the reporting of any SUSARs to the ethics committees (EC), the competent authorities (CA) and the investigators in compliance with applicable laws and regulations, and in accordance with any trial specific agreements between the sponsor and a co-sponsor.

Expedited reporting of SUSARs will occur no later than 15 days after HOVON Data Center had first knowledge of the serious adverse event. For fatal or life-threatening cases this will be no later than 7 days for a preliminary report, with another 8 days for a complete report.

The manner of SUSAR reporting will be in compliance with the procedures of the ethics committees and health authorities involved.

8.5. Reporting special situations

Overdose, abuse, misuse, medication error or occupational exposure are special reporting situations and must be reported to HOVON Data Center immediately.

Please inform HOVON Data Center of these events within 3 days hours after the event was known to the investigator by email (hdc@erasmusmc.nl). Note that these special reporting situations in and of themselves are not AEs. If a special reporting situation results in an SAE, an SAE form should be completed and sent to HOVON Data Center (see section 12.3.1).

8.6. Annual safety report

The annual safety report will be combined with the annual progress report (see chapter 12.4).

In addition to the expedited reporting of SUSARs, the sponsor will submit, once a year throughout the clinical trial, a safety report to the accredited METC, competent authority, and competent authorities of the concerned Member States.

This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;

– a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

8.7. Follow-up of adverse events

All SAEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.

SAEs need to be reported from the first administration of treatment according to protocol until day 14 days after the last dose of the IMP or until the start of subsequent systemic therapy for the disease under study, if earlier.

8.8. Data Safety Monitoring

No data safety monitoring board (DSMB) will be implemented because the investigational product has been very well tolerated in the phase II and III studies and a previous fase III trial did not show any increase in AE or SAE in the anidulafungin voriconazole combination arm compared with the voriconazole monotherapy arm.

9. STATISTICAL ANALYSIS

9.1. Primary endpoint analysis

The primary endpoint is defined as all-cause mortality at 42 days (6 weeks) after randomization. The MITT population is considered the main analysis population.

The relation between randomly allocated treatment and the incidence of the primary endpoint in the MITT population will be described by

- the total number of endpoint events by allocated treatment;
  - a crude, unadjusted odds ratio. Logistic regression analysis will be conducted, with randomly allocated treatment as independent predictor variable and the incidence of the primary endpoint as dependent outcome variable;
  - an adjusted odds ratio. A multivariable logistic regression analysis will be applied. The effect of randomly allocated treatment will be adjusted for age, pulmonary or (also) extrapulmonary disease, ICU admission, baseline serum galactomannan status, post allogeneic stem cell transplantation status, and acute or chronic GVHD for which patients are receiving systemic immunosuppressive therapy.

The adjusted odds ratio will be considered the key primary endpoint analysis

The same analysis will be performed for secondary endpoints 2 to 6. The listed sub-populations will be analyzed using simple and multivariable logistic regression (the adjustment factors - as far as applicable - are listed above). Since these analyses will be considered exploratory only and these endpoints are defined as secondary endpoints, no correction for multiple testing will be performed.

9.2. Secondary endpoints analyses

The 1st secondary endpoint is defined as attributable mortality at 84 days (12 weeks) follow-up, which will be analyzed in the main analysis population. For this analysis, non-attributable mortality will be considered competing risk for attributable mortality

For the analysis of secondary endpoints 2 to 6 see 9.1.

Secondary endpoints 7 and 8 will be analysis using descriptive statistics (6- and 12-week mortality with 95% confidence intervals). If the number of patients in the 2 subgroups described in endpoint 6 and 7 are sufficiently large (□25) we will compare the 6 mortality of both groups using the Fisher-Exact test.

Regarding endpoint 9, several studies have studied the impact of galactomannan kinetics on outcome in patients with IA. Studies have shown a reasonable correlation between galactomannan kinetics in the weeks following the initiation of therapy and outcome[21]. However, several questions remain; What is the optimal timing of the follow-up galactomannan measurement? Is the rate of decline more informative than a simpler binary outcome (decline/no decline or decline with 0.5 OD units)? The statistical analysis plan for this endpoint will be written when the study is completed, and the number of galactomannan positive patients and the number of follow-up plasma samples is known.

Secondary endpoints 10 will be analysis using descriptive statistics (6 and 12-week mortality with 95% confidence intervals).

The 11th secondary endpoint is defined as the total duration of the hospital stay. The relation between randomly allocated treatment and this secondary endpoint will be analyzed using a Mann-Whitney U test.

9.3. Multiplicity

There is only 1 primary endpoint and no interim analysis for efficacy will be performed. Therefore, the primary endpoint will be tested at the α=0.05 level (two-sided test).

9.4. Missing data

Patients that are lost to follow-up after the start of study drugs will be considered treatment failures in the mITT analysis.

9.5. Sample size calculation

In the pivotal trial on voriconazole and anidulafungin combination therapy the mortality in the voriconazole control group was 28% 6 weeks after the start of therapy.[2] This study only included patients with an underlying haematological disease. Furthermore, in a large pragmatic trial with fewer exclusion criteria and in which a small number of ICU patients will be included as well, we expect a somewhat higher mortality of 35%, We consider a 33% lower overall mortality with combination therapy (=from 35% to 23,33%) compared to monotherapy of clinical importance. This leads to a sample size of 237 evaluable patients per group (=included in the mITT population as defined above) to show superiority of combination therapy compared with monotherapy with an alfa of 0.05 and a power of 80%. Follow-up for the primary endpoint is 6 weeks after the start of antifungal therapy.

9.6. Responsibility for data analysis

The coordinating investigator will be responsible for analyzing the study data.

9.7. Monitoring

This trial is part of the HOVON site evaluation visit program. Site evaluation visits will be performed for HOVON trials to review the quality of the site and not specifically the quality of a certain trial. It will enable HOVON to collect quality data and facilitate improvement of the participating sites. Data cleaning or monitoring of the performance of specific trials is not the goal of the site evaluation visits. Site evaluation visits will be performed according to the site evaluation visit plan.

The HOVON site evaluation visit plan applies to sites in the Netherlands and Belgium only. Monitoring of the quality of trial conduct in participating sites from other countries will be organized by the coordinating investigator or co-sponsor. The frequency and content of the site visits in other countries will be at least equal to the specifications of the site evaluation visit plan and are described in a monitoring plan provided by HOVON.

Direct access to source documentation (medical records) must be allowed for the purpose of verifying that the data recorded in the CRF are consistent with the original source data. The sponsor expects that during site visits the relevant investigational staff will be available, the source documentation will be available, and a suitable environment will be provided for review of study-related documents.

9.8. Interim efficacy analysis

An interim efficacy analysis will not be performed. One interim futility analysis will be performed after the inclusion of 50% of the planned sample (see 7.7).

10. COST-EFFECTIVENESS ANALYSIS

The cost-effectivity of combination therapy compared with triazole monotherapy will be analyzed only if a statistical difference (p0.05 or lower) is observed for the primary endpoint or for secondary endpoint 2. The full statistical analysis plan for endpoint 12 is therefore not yet complete but will be developed after the analysis of these endpoints has been completed and in collaboration with prof. dr. C. Uyl-de Groot of the institute of Medical Technology Assessment in Rotterdam (iMTA). The analysis will estimate if combination therapy is cost saving and if this is not the case the cost per quality adjusted life year of combination therapy compared to monotherapy will be calculated. For this, not only quality of life data, the (time to) death but also detailed data on each of the following clinical parameters that are associated with substantial increase in costs will be collected; Duration of hospital stay and number of days admitted to the intensive care unit, blood products and antifungal drugs administered as well as enteral or parenteral nutrition given between baseline and week 12 of follow-up. For the quality of life data collection, the EuroQOL EQ5D-5L questionnaire will be used (including the proxy or telephone version if needed, appendix 5). Because the majority of the patients with IA in our study will consist of patients treated for AML with intensive chemotherapy and of patients that received an allogeneic stem cell transplantation, previously published utility scores from these patient populations will be used to calculate the cost per quality adjusted life years gained (QALY) with combination therapy compared with monotherapy.[19] Apart from quality of life data, we will collect data on loss of income up to 24 weeks after inclusion to allow for an analysis of cost-effectivity from the society perspective. For this purpose, a set of dedicated questionnaires will be used (appendix 5).

The study population will consist of 3 major study populations: Patients receiving chemotherapy for AML, patients with graft-versus-host-disease and other patients. If a significant treatment effect of the intervention is observed in one of these subgroups, an exploratory cost-effectiveness analysis will be done for this subgroup.

11. ETHICAL CONSIDERATIONS

11.1. Regulation statement

The study will be performed in accordance with the protocol, the guidelines of Good Clinical Practice/ICH, which underwrites the principles of the Declaration of Helsinki, as most recently revised by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013.

11.2. Ethical committee approval

The study protocol will be formally submitted to the ethical committee of the Erasmus MC. The study will start after approval from the ethical committee has been obtained. The nature of the study and an outline of those investigative procedures, which might be in excess of their usual care, will be explained to the patients. They will be required to give their written informed consent before entering the study.

11.3. Recruitment and consent

Patients will be recruited at study sites in the Netherlands and Belgium. It is the responsibility of the investigators or the co-investigators to obtain written informed consent from each subject participating in this study, after adequate explanation of the aims, methods, anticipated, and potential hazards of the study.

Besides the specific information regarding the study, the following standard items are covered in the patient information form (Dutch: patiënten informatie formulier):

- Patient's right to withdraw from the clinical study anytime without giving reasons and without any consequences for further medical treatment.
- The information that all study findings will be stored in a computer database and handled confidentially
- Patient names will be kept separate from research data and patients will be identifiable by subject number only.
- Information about the possibility of inspection of relevant parts of the hospital records by regulatory authorities. Inspection will only take place if a confidentiality agreement has been signed.
- The existence of patient insurance policy in case the patient will be harmed by participating in the study (using the study drug)
- All novel clinically relevant information that will become available during the study and is possibly important for the patient will be communicated to him/her by one of the investigators.

The signature of an investigator or co-investigator on the form will attest that the information in the consent form was accurately explained and understood. Thereafter the patient will sign after a period of reflection. If new safety information results in significant changes in the risk/benefit assessment, the consent form will be reviewed and updated after approval by the ethical committee. Then, all subjects (including those already being treated) will be informed of the new information, will be given a copy of the revised form and will be asked to give their consent to continue the study.

11.4. Benefits and risks assessment

Anidulafungin has been registered and used in the Netherlands for the treatment of invasive candida infections for >10 years and is considered a very safe drug. Also, the safety of the combination of anidulafungin and voriconazole that the patients in the intervention group will receive has previously been demonstrated in a large randomization clinical trial.[2] The risks are therefore considered very low.

If the improved mortality with combination therapy that was observed in the study by Marr K et al. is confirmed in our study, the patients in the study as well as future patients may potentially benefit from this combination treatment.

Hepatic metabolism of anidulafungin has not been observed and anidulafungin is not a clinically relevant substrate, inducer, or inhibitor of cytochrome P450 (CYP450) isoenzymes. It is therefore very unlikely that anidulafungin will have significant drug-drug interactions with concomitant medication taken by the patient.

As a result of the underlying disease as well as the chemotherapy, serious adverse events are very frequently observed in this patient population (e.g. bleeding, life threatening infections, death due to progression of the underlying disease). The study will comprise of 4 study visits and as most patients will be hospitalized at the start of therapy few of these will be additional hospital visits on top of the standard of care.

11.5. Compensation for injury

Liability insurance sponsor/investigator

The sponsor has a liability insurance in place for the Dutch study sites in accordance with article 7, subsection 6 of the WMO.

The Belgian coordinating party has a liability insurance in place for the Belgian sites in accordance with Belgian legislation.

Insurance for study participants

The Erasmus MC WMO insurance applies for all patients included in one of the Dutch study sites. The certificate can be found in appendix 4a.
The UZ Gasthuisberg insurance (also called the “no-fault aansprakelijkheidsverzekering”) applies for all patients included in one of the Belgian study sites. The certificate can be found in appendix 4b.

11.6. Incentives

No incentive will be given.

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1. Handling and storage of data and documents

Data will be handled confidential and if possible, anonymously. Where it is necessary to be able to trace data to an individual subject, a subject identification code list will be used to link the data to the subject. The code will not be based on the patient initials and birthdate. The key to the code will be safeguarded by the investigator. as the data and human material will be kept for a longer period of time. The handling of personal data will comply with the Belgian and Dutch Personal Data Protection Act (in Dutch: De Wet Bescherming Persoonsgegevens, Wbp).

Case record form (CRF)

All data of patients, including results from standard procedures during treatment, collected during the study will be recorded in Case Record Forms. The CRF must be completed fully and legibly. Corrections of possibly erroneous entries must be carried out in such a manner that the initial entry is not rendered illegible. Corrections should be written alongside or above the pertinent place with the date and initials. Correction fluid must not be used.

The investigators are responsible for the quality of the data recorded in the Case Record Forms (CRF). Where the investigators have not been responsible for completing the CRF, an additional signature from the co-investigator overseeing the data entry of the study must be obtained.

In the event that the investigators need to deviate from the protocol, the nature of and reasons for protocol deviation must be recorded in the hospital patient record and in the CRF. In nearly all cases it is desirable that the patient continues the study to allow the most informative intention-to-treat analysis; This does not mean that the treatment to which the patient was randomized needs to be continued. As illustrated by the flow diagram of the study (figure 1) there can be good reasons to change antifungal therapy after randomization. However, also for patients that go off study for the 3 reasons mentioned in figure 1, a limited number of data will be collected in the CRF (e.g. antifungal therapy, survival)

Privacy rules

Patients will be identified in the CRF by their identification code. The investigators will keep a patient identification log, including sufficient information to link the hospital record and CRFs.

The subjects will be informed that the data will be stored electronically, that local regulations for the handling of computerized data will be followed as described in the written patient information / consent form and that identification of individual

patient data will only be possible for the investigators. Furthermore, the subjects will be informed about the possibility of inspections of relevant parts of the hospital records by health authorities. These officials will be identified and have signed a confidentiality agreement. The data are processed and stored using dedicated GCP compliant electronic CRF (ALEA). From this database the data will be transferred to a statistical program for further analysis. Only data, with coded patient identity will be transferred to the statistician for analysis.

Data processing
After a visual plausibility check the CRF data will be entered in the computer and processed using dedicated GCP compliant electronic CRF (ALEA). When all data have been approved by the local investigator, the database will be locked for that site and the data can be transferred from the database to a statistical data file, with conversion in uniform data and formation of a master file for further analysis. The data will also be approved by the investigator and locked after approval at the time when a patient moves from one hospital to another

Data achieving
Patient identification log, hospital records, informed consent forms, case record forms and databases must be kept for at least 15 years after completing the study. If the investigators move or retire, they must nominate someone in writing to be responsible for record keeping. Archived data may be held on microfiche or electronic record, provided that a backup exists, and a hard copy can be obtained from it if required.

12.2. Monitoring and Quality Assurance

Please refer to our monitoring plan.

12.3. Amendments

Amendments are changes made to the research after a favorable opinion by the accredited METC/IRB has been given. All amendments will be notified to the METC/IRB that gave a favorable opinion.

A ‘substantial amendment’ is defined as an amendment to the terms of the METC/IRB application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:
- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC/IRB and to the competent authority.

Non-substantial amendments will not be notified to the accredited METC/IRB and the competent authority but will be recorded and filed by the sponsor. Examples of non-substantial amendments are typing errors and administrative changes like changes in names, telephone numbers and other contact details of involved persons mentioned in the submitted study documentation.

12.4. Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC/IRB once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5. End of study report

The sponsor will notify the accredited METC/IRB and the competent authority of the end of the study within a period of 90 days. The end of the study is defined as the last patient's last visit.

In case the study is ended prematurely, the sponsor will notify the accredited METC/IRB and the competent authority within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC/IRB and the Competent Authority.

12.6. Public disclosure and publication policy

The sponsor is free to publicly disclose and publish all research data. Please refer to the contract between de sponsor and the subsidizing party for arrangements made concerning public disclose and publication of research data.

13. STRUCTURED RISK ANALYSIS

13.1. Potential issues of concern

a. Level of knowledge about mechanism of action

While voriconazole and other azoles inhibit fungal cell membrane synthesis, the echinocandins block production of 1,3-beta-D glucan, a key component of fungal cell walls.

Combination therapy with voriconazole and an echinocandin is an intriguing possibility given the different mechanisms of action of these two agents.

b. Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism
See SPCs submitted with this protocol

c. Can the primary or secondary mechanism be induced in animals and/or in ex-vivo human cell material?
The purpose of this study is showing efficacy of combination therapy in patients with IA. In a neutropenic rabbit model of invasive pulmonary aspergillosis, the combination of voriconazole and anidulafungin was superior to single agent therapy with respect to mean pulmonary fungal burden and survival, among other measures.[16]

d. Selectivity of the mechanism to target tissue in animals and/or human beings
Voriconazole has been shown to be more selective for fungal cytochrome P-450 enzymes than for various mammalian cytochrome P-450 enzyme systems. Anidulafungin inhibits the synthesis of beta (1,3)-D-glucan, an essential component of the cell wall of many filamentous fungi and yeast. Beta (1,3)-D-glucan is not present in mammalian cells.

e. Analysis of potential effect
The potential positive effects of combination therapy are described in 6.1 and 6.2

f. Pharmacokinetic considerations
Pharmacokinetics of voriconazole/isavuconazole/isavuconazole and anidulafungin are well known and described in the SPCs submitted with this protocol. There are no drug-drug interactions between both drugs.

g. Study population
Immunocompromised patients with a suspected, probable or proven IA as well as ICU patients admitted with influenza diagnosed with IA according to the in -and exclusion criteria in the protocol

h. Interaction with other products
Voriconazole and isavuconazole or posaconazole have no significant drug-drug interactions with anidulafungin. Anidulafungin is not metabolized by the liver and is not renally excreted. Hepatic metabolism of anidulafungin has not been observed and anidulafungin is not a clinically relevant substrate, inducer, or inhibitor of cytochrome P450 (CYP450)

isoenzymes. It is unlikely that anidulafungin will have clinically relevant effects on the metabolism of drugs metabolized by CYP450 isoenzymes.

Anidulafungin undergoes slow chemical degradation at physiologic temperature and pH to a ring-opened peptide that lacks antifungal activity. The in vitro degradation half-life of anidulafungin under physiologic conditions is about 24 hours. In vivo, the ring-opened product is subsequently converted to peptidic degradants and eliminated.

i.Predictability of effect

There are no predictable side-effects of anidulafungin therapy. The effect of combination therapy on the survival in patients with IA is currently not predictable as it has been studied in only 1 randomized clinical trial and this trial was inconclusive.

j. Can effects be managed?

Not applicable

13.2. Synthesis

In conclusion, we think that in this study the potential benefits outweight the risks as the drug that is being used is a registered drug that has been used extensively worldwide, has an overall good safety profile and has no drug-drug interactions.

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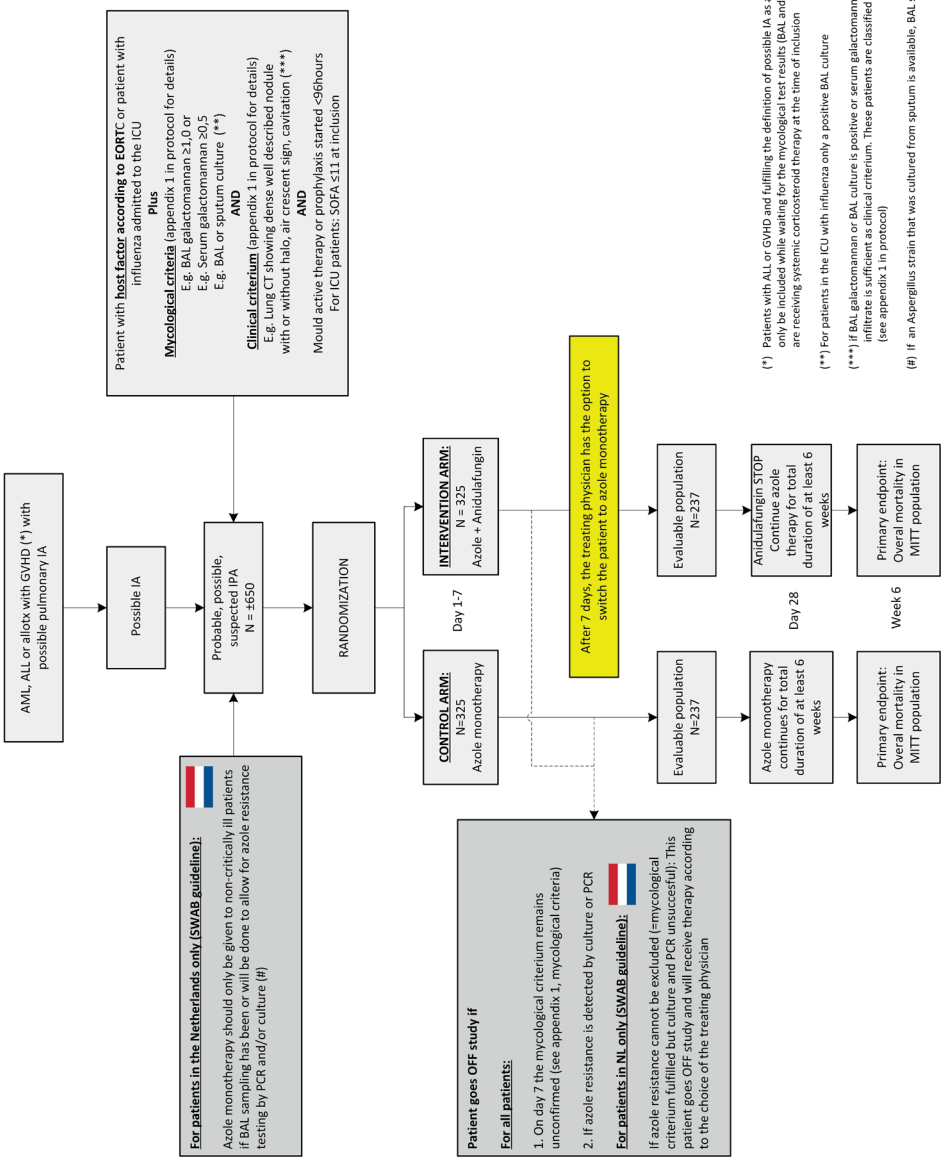

Figure 1. Flow diagram

APPENDIX 1: MODIFIED EORTC/MSG CONSENSUS DEFINITIONS FOR DIAGNOSIS OF PROVEN, PROBABLE, POSSIBLE OR SUSPECTED INVASIVE ASPERGILLOSIS.

Proven invasive aspergillosis

Histopathologic, cytopathologic, or direct microscopic examination of a needle aspiration or biopsy specimen showing hyphal forms with evidence of associated tissue damage (either microscopically or as an infiltrate or lesion by imaging) in combination with a positive aspergillus PCR on the sample

OR

Recovery of a mould by culture from a sample obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding BAL, cranial sinus cavity, and urine.

Probable invasive aspergillosis

Defined by at least:

- One host factor (See below)
  AND
- One clinical criterion (See below)
  AND
- One mycological criterion (See below)

Possible invasive aspergillosis

Defined by at least:

- One host factor (See below)
  AND
- One clinical criterion (See below)

Suspected invasive pulmonary aspergillosis

Please note that the EORTC/MSG classification does not include suspected invasive pulmonary aspergillosis in its classification

Defined by at least:

- One host factor (See below)
  AND
- A pulmonary infiltrate other than a nodule, halo sign, cavity or air-crescent sign
  AND
- One mycological criterion (See below)

Host factors

1. Recent history of neutropenia (<0.5 x 109/L (<500 neutrophils/mm3) for >10 days) temporally related to the onset of fungal disease or ongoing neutropenia; Patients with a newly diagnosed AML can be considered to be neutropenic for at least 10 days and therefore fulfill this criterium also at the time of AML diagnosis.
2. Receipt of an allogeneic stem cell transplant;
3. Prolonged use of corticosteroids (excluding patients with ABPA) at an average minimum dose of 0.3 mg/kg/day prednisone equivalent for >3 weeks;
4. Treatment with other recognized T-cell immune suppressants such as ciclosporin, TNF-α blockers, specific monoclonal antibodies (such as alemtuzumab), or nucleoside analogues during the past 90 days;
5. Inherited severe immunodeficiency (eg, chronic granulomatous disease, severe combined immunodeficiency).

Clinical criteria

Must be consistent with the microbiological findings, if any, and must be temporally related to current episode. Every reasonable attempt should be made to exclude an alternative etiology.

1. Lower respiratory tract fungal disease

The presence of one of the following three signs on CT:

- Dense, well-circumscribed lesion with or without a halo sign;
- Air crescent sign;
- Cavity.
- For patients with a serum galactomannan value of 0.5 or higher or for patients with a BAL galactomannan of 1.0 or higher, the presence of any pulmonary infiltrate is considered sufficient evidence of lower respiratory tract fungal disease

2. Tracheobronchitis:

Tracheobronchial ulceration, nodule, pseudomembrane, plaque or eschar seen on bronchoscopy.

3. Sinonasal infection

Imaging showing sinusitis PLUS at least one of the following:

- Acute localized pain (including pain radiating to the eye);
- Nasal ulcer with black eschar;
- Extension from the paranasal sinus across bony barriers, including into the orbit.

4. CNS infection

At least one of the following:

- Focal lesions on imaging;
- Meningeal enhancement on MRI or CT.

MYCOLOGICAL CRITERIA

1. Cytology, direct microscopy or culture:

- Sputum, BAL and bronchial brush samples demonstrating the presence of fungal elements either by recovery by culture of Aspergillus spp. or detection by cytology or
- direct microscopy of hyphal forms in combination with a positive aspergillus PCR on sputum, BAL or bronchial brush
- Sinus aspirate: recovery by culture of Aspergillus spp. from aspirate *or* the detection of hyphal forms by cytology or microscopy in combination with a positive aspergillus PCR on the aspirate.
- Skin ulcers, draining soft tissue lesions or fissure for which both a positive microscopy (hyphae) and positive Aspergillus culture are required.

2. Detection of galactomannan antigen or DNA of aspergillus defined as one of the following:

- Galactomannan antigen EIA (Platelia):
  1. Serum sample positive for galactomannan (0.5 or higher);
  2. BAL sample positive for galactomannan (1.0 or higher).
- PCR:
  1. Positive Aspergenius spp. PCR on BAL fluid (cycle threshold 38 or lower) in combination with a galactomannan BAL OD value of 0.5-0.9 is considered a positive mycological criterium in this study
  2. Positive Aspergillus spp. PCR on sputum, BAL or bronchial brush sample in combination with hyphal forms detected by cytology or direct microscopy

NOTE: Positive aspergillus PCR results alone will NOT be considered sufficient mycological evidence of invasive fungal disease.

APPENDIX 2: PATIENT VISIT SCHEDULE

	Screening	Baseline (D1)	D2-6	D3	D5	D7	D8-D28	D14	D28	D42	D84	D168	D8-28
Eligibility check[(#)]	x	x [(#)]											
Informed consent		x											
Study drug administration[(^)]		x	x			x	(x)						x
Medical history		x											
Serum sampling[(*)]		x		x	x	x		x	x				
Patiënt details		x											
Register use of any antifungals		x						x	x	x	x	x	
Register use of TPV or EN after baseline visit										x		x	
Register use of any blood products administered after baseline visit										x		x	
Register hospital and ICU days from baseline to week 24										x		x	
Quality of life questionnaire		x								x	x	x	
Loss of income questionnaire		x									x	x	
Neutropenic status		x				x		x	x	x	x		
Survival status						x		x	x	x	x	x	

Please note that few exceptions notwithstanding, screening and baseline visit will be done on the same day.

Gray columns indicate the days that a hospital visit is required: Although the large majority of the patients will be in the hospital during the first week of therapy some patients will be outpatients. Patients who are outpatients and are included in the study are required to visit the outpatient clinic for study drug administration up until day 7.

Please note that, except for the screening and baseline visit, none of the other visits require an additional patient visit to the hospital as all these data can be collected from the patient files and by contacting the patient or the general practitioner or relatives of the patient.

[(^)] For the patients randomized in the combination therapy group. The minimum duration of study drug treatment is 7 days. After day 7 and up until day 28 the investigator decides if the treatment needs to be continued for a maximum of 28 days.

(#) For ICU patients the SOFA score should be recalculated if >24hrs pass between screening and baseline and patient excluded if SOFA has increased to >11 points.

(*) This should only be done if the patient is still in the hospital or visiting the outpatient clinic at these study dates. Please note that the day 14 and day 28 sampling can be done between day 11 and 17 and day 25 and 31 respectively (so day 14±3days and day 29±3days). The standard operating procedure regarding serum sampling and storage of serum by the lab is described in appendix 6 in more detail.

TPV= total parenteral nutrition. EN= enteral nutrition.

Chapter 12

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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Chapter 13

Nederlandse samenvatting

In Nederland vraag je met een consult geestelijke gezondheidszorg geen advies psychiatrie.

INTRODUCTIE

Jaarlijks sterven nagenoeg 1.5 miljoen mensen aan invasieve schimmelinfecties, veroorzaakt door gisten of draadvormige schimmels. Een voorbeeld van een gist is *candida*. De belangrijkste vertegenwoordiger van de draadvormige schimmel is *Aspergillus*. Bij patiënten met een sterk verminderde afweer kan de Aspergillus schimmel een gevaarlijke invasieve infectie veroorzaken. We noemen deze ziekte invasieve aspergillose. Een verminderde afweer kan het gevolg zijn van het gebruik van afweer onderdrukkende medicijnen. Die medicijnen worden gebruikt om afstoting te voorkomen na stamcel- of orgaantransplantatie. Invasieve aspergillose kan ook optreden naar aanleiding van een tekort aan witte bloedcellen, zoals dat bijvoorbeeld veroorzaakt kan zijn door chemotherapie. Jammer genoeg zien we het aantal patiënten met invasieve aspergillose de laatste decennia sterk stijgen. Patiënten die langdurige immuunsysteem-onderdrukkende medicijnen nemen, leven dankzij de vooruitgang van de geneeskunde langer dan voorheen. Daardoor lopen ze ook meer risico om bepaalde “opportunistische” infecties op te lopen.

Er zijn verschillende soorten *Aspergillus*. Meestal wordt invasieve aspergillose veroorzaakt door de *Aspergillus fumigatus* variant. *Aspergillus fumigatus* komt vaak voor in het milieu en verspreidt zich als “spore” door de lucht. Deze spore kan worden ingeademd. Bij mensen met een goede afweer wordt deze schimmel snel opgeruimd. Bij sterk verminderde afweer kan de schimmel beginnen woekeren en schade berokkenen. Deze infectie begint dan ook meestal in de longen. Een schimmelinfectie in de longen veroorzaakt koorts, hoesten, pijn bij de ademhaling en kortademigheid. Soms is het moeilijk te bewijzen dat een patiënt een infectie met deze schimmel heeft opgelopen. Het stellen van de diagnose gebeurt aan de hand van specifieke criteria, opgesteld zijn door experten. Deze criteria worden de gereviseerde criteria van de European Organization for Research and Treatment of Cancer / Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG)” genoemd. Een schimmelinfectie, of het vermoeden van een schimmelinfectie omschrijft men met de termen “proven” of bewezen, “probable” of waarschijnlijk en “possible” of mogelijk.

Men spreekt van een bewezen schimmelinfectie als er tekenen daarvan gevonden worden in een weefselbiopt (dit is een stukje weefselmateriaal van de patiënt, dat meestal na aanprikken of wegname van een verdacht letsel wordt verkregen) of als de schimmel kan gekweekt worden uit steriel bekomen lichaamsmateriaal. Het nemen van een biopt is vaak niet mogelijk omdat de patiënt te ziek is en de kans op mogelijke complicaties, zoals het prikken van een klaplong of het optreden van een ernstige nabloeding, te groot is. Daarom is de diagnose bijna altijd een waarschijnlijke of mogelijke schimmelinfectie.

Om die diagnose te stellen, moet aan drie voorwaarden zijn voldaan: ten eerste moet er sprake zijn van een vatbare patiënt ten gevolge van een onderdrukt immuunsysteem, zoals na een stamceltransplantatie of bij acute leukemie. Ten tweede moet voldaan zijn aan het klinisch criterium: beeldvorming, zoals CT-scan, moet het typisch teken van schimmelinfectie vertonen, met name een nodule (of knobbeltje) met daarrond een halo-teken. Ten derde moet er ook microbiologisch bewijs zijn: een positieve kweek met *Aspergillus* op een niet steriele manier bekomen, zoals door kweek op slijm, afkomstig van de bovenste of onderste luchtwegen. Helaas zijn de meeste invasieve aspergillosen zeer moeilijk te kweken en moet het bewijs vaak op een andere manier worden aangeleverd. Dit kan door diagnostische testen die indirect bewijs leveren voor de aanwezigheid van een schimmelinfectie of door het aantonen van antistoffen tegen een deel van de celmembraan van *Aspergillus*, zoals tegen het suikerbestanddeel galactomannan, of tegen Bèta2-D-glucan. Vaak zijn patiënten niet in staat om het vereiste slijm op te hoesten, waardoor het nodige materiaal diep in de long dient te worden verzameld door middel van longspoeling, ook “broncho-alveolaire lavage” (BAL) genoemd. Een longspoeling gebeurt aan de hand van een bronchoscopie (endoscopie in de longen). Het longspoelvocht of “BAL-vocht” wordt verder onderzocht voor kweek en er wordt nagegaan of er schimmelmateriaal in het vocht kan worden gevonden. Dit schimmelmateriaal kan een deel van de celmembraan zijn of genetisch materiaal van de schimmel.

AZOLE-RESISTENTE ASPERGILLOSE

Invasieve aspergillose moet snel behandeld worden met een antischimmel medicijn. Er zijn vier klassen antischimmelmedicijnen: azoles, echinocandines, polyenen en fluorpyrimidines. Al meer dan 15 jaar bestaat de voorkeursbehandeling uit de klasse van de azoles: voriconazole of isavuconazole. Ondanks deze behandeling overlijden nog steeds 20 tot 30% van de patiënten binnen de 12 weken na de diagnose. Bovendien is er in Nederland, maar recenter ook in België, meer en meer sprake van resistentie tegen de antischimmelmedicijnen uit de klasse azoles zoals voriconazole. Daarmee wordt bedoeld dat de *Aspergillus* minder gevoelig of zelfs ongevoelig is voor de behandeling. Resistentie wordt soms vastgesteld bij patiënten die langdurig behandeld worden met antischimmelmedicijnen. Anderzijds wordt aangenomen dat het gebruik van antischimmelmiddelen in de landbouw de belangrijkste oorzaak is van resistentie. De aangewende antischimmelmiddelen in de landbouw lijken zeer goed op de antischimmelmedicijnen die gebruikt worden om patiënten met invasieve aspergillose te behandelen. Schimmels wapenen zich door hun genetisch materiaal aan te passen, wat hen toelaat toch te groeien wanneer ze worden blootgesteld aan deze schimmelmedicijnen. Het inademen

van een spore van een azole-resistente schimmel kan leiden tot een azole-resistente invasieve schimmelinfectie.

Uit een aantal studies is gebleken dat patiënten, geïnfecteerd met azole-resistente aspergillose, in 50 tot 88% van de gevallen overlijden. Het aantal patiënten beschreven in deze studies is echter zeer beperkt, waardoor het moeilijk is om daaruit relevante conclusies te trekken. Om te onderzoeken of het vinden van een azole-resistente schimmel gepaard gaat met een hogere kans op overlijden, hebben we in **hoofdstuk 3** twee groepen patiënten vergeleken met invasieve aspergillose en positieve kweek. Een lijst van patiënten werd bekomen uit 3 ziekenhuizen in Nederland. Deze patiënten werden behandeld tussen 2011 en 2015. De gegevens uit het medische dossier van deze patiënten werden anoniem verwerkt en opgedeeld in twee groepen. De eerste groep bevat de patiënten met invasieve aspergillose gevoelig aan azolen. De tweede groep bestaat uit patiënten met azole-resistente invasieve aspergillose. In totaal werden gegevens van 196 patiënten onderzocht waarvan 159 patiënten in groep 1 en 37 patiënten in groep 2. Uit deze studie bleek dat, 3 maanden na het starten van antischimmelmedicijnen, 62% van patiënten met azole-resistente invasieve aspergillose (groep 2) overleden waren. Dit percentage was veel hoger dan in de groep patiënten met azole-gevoelige aspergillose. In die groep was 3 maanden na het starten van de behandeling 37% van de patiënten niet meer in leven. Met andere woorden: de kans op overlijden is 25% hoger wanneer de verantwoordelijke aspergillus schimmel azole-resistent is. Dit is de grootste studie tot dusver die klaar en duidelijk aantoont dat, wanneer een patiënt geïnfecteerd is met een azole-resistente *Aspergillus fumigatus*, de prognose van de patiënt veel slechter is.

Jammer genoeg is resistentie van *Aspergillus* voor azole het hoogst in Nederland en blijkt dit nu ook in België toe te nemen. Recent werd in Nederland een nieuwe test ontwikkeld waarmee op een snellere manier zowel de aanwezigheid van *Aspergillus* als de gevoeligheid van deze schimmel voor de courant gebruikte "azole" antischimmel medicijnen (voriconazole, posaconazole, itraconazole) kan worden opgespoord in de vloeistof van het longonderzoek. Door deze test, kunnen we snel nagaan of er sprake is van een azole-resistente schimmel.

Om het probleem van azole-resistente aspergillose aan te pakken, hebben we in de zomer van 2016 een bijeenkomst georganiseerd met vertegenwoordiging uit alle universitaire centra in Nederland, waarbij een microbioloog, hematoloog en infectioloog betrokken waren. Daarvoor hebben we als uitgangspunt naar elk centrum een enquête gestuurd met de bedoeling te inventariseren hoe invasieve aspergillose in het betrokken centrum wordt behandeld. De verschillen werden samengevat en er werd een gemeenschappelijk behandelplan afgesproken. De enquête is samengevat in **hoofdstuk 2** en toonde aan dat er best wat verschillen zijn in de manier waarop invasieve aspergillose in de Nederlandse academische centra wordt behandeld. Het principe van

dit behandelplan proberen we in de volgende alinea kort samen te vatten zonder al te veel in detail te treden.

Bij vermoeden van invasieve schimmelinfectie bij patiënten met onderliggende bloedkanker, zoals leukemie, wordt een longspoeling of een broncho-aveolaire lavage (BAL) verricht in een poging microbiologisch bewijs te vinden voor invasieve schimmelinfectie. Na de longspoeling wordt de behandeling gestart met een azole (voriconazole, posaconazole of isavuconazole). Het longspoelsel wordt ondertussen onderzocht op genetisch materiaal van *Aspergillus fumigatus*. Als genetisch materiaal van de schimmel wordt gedetecteerd, wordt nagegaan of er bepaalde mutaties aanwezig zijn waarvan we weten dat ze gevonden worden bij azole-resistente *Aspergillus* schimmel. Als er resistentie wordt vastgesteld, hetzij door kweek, hetzij door deze genetische test op schimmel DNA, dan wordt de behandeling gewijzigd naar een ander schimmelmedicijn uit de klasse polyenen, met name liposomaal-amfotericine B. Volgens een internationaal expertenpanel kan je azole-resistente aspergillose enkel met dit medicijn behandelen.

Dit behandelplan wordt geëvalueerd in de Azole-Resistance MANagement Study (AzoRMan-study) in de hoop de overleving van patiënten met azole-resistente aspergillose te verbeteren door azole-resistentie sneller vast te stellen en snel de correcte behandeling te starten. Deze studie laat ook toe in te schatten hoe vaak een patiënt geïnfecteerd is met een invasieve aspergillose die azole-resistent is. In december 2019, zijn er ruim 200 patiënten geïncludeerd in de studie. De tussentijdse resultaten zijn te lezen in **hoofdstuk 9**. Tot dusver is er bij minder dan de helft van de patiënten microbiologisch bewijs van invasieve aspergillose te vinden. Tot op heden zijn er bij 4 patiënten (2%) tekenen van azole-resistente aspergillose gevonden. Dat betekent dat ongeveer 8.5% van alle patiënten met invasieve aspergillose geïnfecteerd is met een azole-resistente *Aspergillus* stam. De genetische test die resistentie opspoort, is helaas niet altijd succesvol. Het zal nog even duren tot deze studie klaar is en er definitieve conclusies kunnen getrokken worden.

Jammer genoeg kan liposomaal-amfotericine B enkel toegediend worden via infuus. Er is geen afdoende orale vorm (inname via de mond) voorhanden. Bovendien kan dit geneesmiddel gepaard gaan met soms ernstige bijwerkingen, zoals een allergische reactie, ernstige nierfunctiestoornissen en stoornissen van de zouten in het lichaam. Een behandeling moet in de regel verder gezet worden tot de patiënt geen longafwijkingen meer vertoont op beeldvorming en tot de patiënt geen immuunsysteem onderdrukkende medicatie meer neemt. Vaak duurt het maanden vooraleer een behandeling met antischimmelmedicijnen kan worden gestaakt, waardoor de patiënt langdurig in het ziekenhuis moet blijven. Dit niet alleen om het geneesmiddel toe te dienen, maar ook om mogelijke bijwerkingen tijdig op te sporen aan de hand van regelmatige bloedanalyses ter controle van nierfunctie en zouten, en andere orgaansystemen. Als het laboratorium erin geslaagd is om de *Aspergillus* schimmel van de patiënt te kweken dan

kan de gevoeligheid van deze kweek voor verschillende antischimmelmedicijnen getest worden. Dit gebeurt in de regel door nationale schimmel referentie laboratoria. Daar wordt nagegaan met welke antischimmelmedicijnen de groei van *Aspergillus* kan worden gestopt. Soms is het zo dat een azole-resistente schimmel kan gedood worden met een antischimmelmedicijn, mits die in een hoge dosis wordt gegeven. Uit dierexperimenteel onderzoek is gebleken dat sommige invasieve azole-resistente aspergillosen behandeld kunnen worden met hogere dosissen van posaconazole, een antischimmelmedicijn uit de klasse van de azolen, dat ook onder de vorm van pillen kan ingenomen worden. Het Erasmus Medisch Centrum en Radboud UMC hebben ervaring met de behandeling van patiënten met een hogere dosis van posaconazole. Om deze ervaring te delen, hebben we in **hoofdstuk 4** de gegevens gerapporteerd van 16 patiënten die behandeld zijn met hoge dosissen posaconazole en pluisden we de dossiers van deze patiënten uit om te kunnen inschatten hoeveel bijwerkingen deze behandeling heeft gehad. Bij 5 van de 16 patiënten hebben we ernstige bijwerkingen gevonden die mogelijk verband hielden met het geven van posaconazole in hoge dosis. Deze konden in de regel goed worden opgevangen zonder het medicijn te moeten stoppen.

Sommige patiënten hebben hoge bloedspiegels, ook al slikken ze de gewone dosis. Daarom hebben we van 25 patiënten met spontaan hoge bloedspiegels bij normale dosering het medisch dossier doorgenomen, op zoek naar eventuele bijwerkingen ten gevolge van de verhoogde blootstelling aan dit middel. Bij vijf patiënten waren ernstige bijwerkingen te noteren en werd de dosis van dit geneesmiddel verminderd. Uit ons onderzoek blijkt dat patiënten op een veilige manier met dit geneesmiddel met een hoge dosis kunnen worden behandeld mits regelmatige controle van de bloedspiegels van het geneesmiddel en regelmatige bloedonderzoek om eventuele bijwerkingen snel te kunnen vaststellen. Uiteraard komen voor de behandeling met posaconazole enkel patiënten in aanmerking die een bewezen verbetering hebben gehad met liposomaal-amfotericine B of patiënten die dit laatste geneesmiddel omwille van onaanvaardbare bijwerkingen hebben moeten staken. De bijwerkingen van posaconazole zijn aanvaardbaar omdat de schimmelinfectie op zich een levensbedreigende infectie is en dus een goede behandeling vereist.

Zoals reeds aangehaald, vergt een invasieve aspergillose vaak een zeer langdurige behandeling. Azole-resistente invasieve aspergillose kan soms behandeld worden met hoge dosissen posaconazole onder de vorm van comprimés, althans nadat de infectie vooraf goed onder controle is gebracht met liposomaal-amfotericine B. Liposomaal-amfotericine B kan ook, bij goede respons op een conventionele dagelijkse toediening, na verloop van tijd in plaats van dagelijks twee- of driemaal per week toegediend worden, waardoor de patiënt ook niet meer elke dag naar het ziekenhuis moet. Liposomaal-amfotericine B stapelt immers op in het lichaam als je het een aantal keer na elkaar hebt toegediend. In een paar centra, zoals LUMC en Erasmus MC, heeft men

enige ervaring met deze manier van behandelen. We hebben van 18 patiënten, die op deze manier behandeld zijn, de gegevens verzameld. We hebben onderzocht of er bij deze patiënten onverwachte bijwerkingen optreden en of de behandeling ook een goed therapeutisch effect heeft gehad. Dit hebben we samengevat in **hoofdstuk 5**. Uit ons onderzoek blijkt dat dit een veilige optie is, ook al moet men bedacht zijn op eventuele nierfunctiestoornissen. Zowel posaconazole in hoge dosis als liposomaal-amfotericine B in een frequentie van twee- tot driemaal per week, maken deel uit van het behandelprotocol dat nationaal is geïmplementeerd in alle Nederlandse academische ziekenhuizen. Deze behandelopties kunnen, zoals vermeld, enkel overwogen worden als de patiënt met azole-resistente aspergillose een goede verbetering heeft getoond met dagelijkse toediening van liposomaal-amfotericine B.

Invasieve aspergillose veroorzaakt niet alleen schimmelinfecties in de longen, maar kan ook leiden tot ernstige infecties op andere plaatsen in het lichaam, zoals in de hersenen. Een schimmelbol in de hersenen verstoort de werking van het hersenweefsel en kan ernstige symptomen veroorzaken zoals epilepsie en zenuwuitval. Ook dit wordt behandeld met azoles, maar als een patiënt geïnfecteerd is met een azole-resistente *Aspergillus* in de hersenen, dan is de behandeling veel complexer vermits liposomaal-amfotericine B slecht in de hersenen doordringt. Deze gelukkig zeldzame complicatie is bijna altijd fataal. Onze ervaring, waarbij we 3 patiënten hebben behandeld met liposomaal-amfotericine B, toegediend in het hersenvocht en dit via een onderhuids reservoir dat rechtstreeks in verbinding staat met een hersenkamer of ventrikel in het brein, hebben we beschreven in **hoofdstuk 6**. Deze behandeling werd gestart omdat de algemene toestand van deze patiënten slechter werd niettegenstaande het toedienen van antischimmelmedicijnen via de normale route. Bij deze patiënten werd de normale behandeling verdergezet en werd bijkomend liposomaal-amfotericine B in het hersenvocht toegediend. Met deze behandeling hebben de 3 patiënten het overleefd. Het is niet duidelijk of deze onconventionele behandeling er echt heeft toe geleid dat de toestand van deze patiënten is verbeterd, maar het is wel aannemelijk dat het geholpen heeft. Omdat er tot dusver geen gegevens zijn gekend, hebben we, samen met collega's van LUMC en Radboud UMC, onze ervaring als een mogelijke behandeloptie wanneer er geen andere behandelmogelijkheden meer zijn omschreven in **hoofdstuk 6**.

Sommige patiënten kunnen geïnfecteerd zijn met verschillende Aspergillus stammen. Een patiënt kan bijvoorbeeld tegelijkertijd geïnfecteerd zijn met een azole-gevoelige en een azole-resistente schimmel. Om dit aan te tonen, zouden verschillende kweken met *Aspergillus* uit de long moeten worden bekomen en zou de gevoeligheidsbepaling voor de verschillende kweken een resultaat moeten opleveren, waarbij de ene kweek gevoeligheid toont voor azoles, terwijl de andere resistentie toont. Om dit vast te stellen, moet er een kweek beschikbaar zijn, maar jammer genoeg is de kweek in de minderheid van de gevallen positief. In **hoofdstuk 8** beschrijven we hoe je met een

test voor schimmel DNA kan aantonen dat een patiënt geïnfecteerd is door zowel een azole-gevoelige als een azole-resistente Aspergillus. Dit moet toelaten de patiënt van in het begin met het juiste antischimmelmedicijn te behandelen.

DIAGNOSE VAN INVASIEVE ASPERGILLOSE

We hebben hierboven al uitgebreid uitgelegd hoe de diagnose van invasieve aspergillose wordt gesteld. Om over een "mogelijke" invasieve aspergillose infectie te spreken, heb je indirect bewijs nodig van de aanwezigheid van invasieve aspergillose. Zo heb je testen die celwandbestanddelen aantonen of schimmel DNA detecteren. Deze onderzoeken vragen best wel wat werk en tijd in het laboratorium. Er is een nieuwe test ontwikkeld die snel kan worden uitgevoerd en heel snel resultaat geeft wanneer het wordt onderzocht op longvocht. Deze test heet in het Engels lateral flow device (LFD) en kan afgelezen worden net als een zwangerschapstest. Het detecteert de aanwezigheid van een eiwit dat Aspergillus vrijstelt als het actief groeit. In **hoofdstuk 7** hebben we deze LFD test uitgeprobeerd op restmateriaal van patiënten met en zonder invasieve aspergillose. Dit restmateriaal is longvocht of BAL vocht en wordt ingevroren aan -20°C, zodat nieuwe testen kunnen worden beoordeeld naar betrouwbaarheid. Zo hebben we deze test verricht op BAL vocht van 247 patiënten, waarvan 79 een zekere of mogelijke invasieve aspergillose hadden. Uit onze studie blijkt dat deze test goede resultaten oplevert. Zo geeft dit een positief resultaat bij 82% van de patiënten met invasieve aspergillose en een negatief resultaat in 86% van de patiënten die geen invasieve aspergillose hebben volgens de geldende definities. Als een digitaal toestel wordt gebruikt om het testresultaat te beoordelen, dan is een negatief resultaat van de test betrouwbaarder omdat de test dan negatief is in 96% van de patiënten die geen aspergillose blijken te hebben. Deze nieuwe test is een mooie aanvulling om snel de diagnose van invasieve aspergillose te stellen.

INFLUENZA-GEASSOCIEERDE INVASIEVE ASPERGILLOSE

Jaarlijks worden meerdere patiënten met een ernstige longinfectie veroorzaakt door het influenzavirus (wetenschappelijk naam voor griepvirus) op de intensive care opgenomen. Het is al lang bekend dat een griepinfectie soms verwikkeld wordt door bacteriële infectie. In 2012 is er een studie verschenen aangaande 40 patiënten die met een longontsteking door griep werden opgenomen op de intensive care van het Universitaire Ziekenhuis van Leuven. Bij 9 van deze patiënten (23%) werd invasieve aspergillose gevonden. Dit is opmerkelijk vermits invasieve aspergillose, zoals reeds

aangehaald, bij patiënten voorkomt met een onderdrukt immuunsysteem. Om vast te stellen hoe vaak invasieve aspergillose als complicatie voorkomt bij patiënten, opgenomen op de intensive care met een longontsteking door griep, hebben we volgend onderzoek verricht: we hebben het dossier van een groot aantal patiënten onderzocht die met longontsteking door griep opgenomen waren op de intensive care afdelingen van zeven verschillende ziekenhuizen in België en Nederland. We hebben onderzocht hoeveel van deze patiënten invasieve aspergillose hebben ontwikkeld. Dit is beschreven **in hoofdstuk 10.1**. We hebben invasieve aspergillose vastgesteld bij 83 van de 432 onderzochte patiënten, wat 19% is, en dit bleek vaak voor te komen bij elk griepseizoen. Er zijn verschillende soorten griepvirus en er blijkt geen verband te zijn tussen het soort griepvirus en de kans om een bijkomende invasieve aspergillose te ontwikkelen. Deze complicatie doet zich ongeveer 2 dagen na het vaststellen van de infectie door griepvirus voor. Het is tot dusver onduidelijk waarom deze complicatie optreedt, maar mogelijk spelen er meerdere factoren mee. We vermoeden dat griep in ernstige vormen het immuunsysteem verzwakt en het longslijmvlies beschadigt, waardoor de schimmel makkelijker kan toeslaan. Maar het zou net zo goed kunnen dat bepaalde mensen genetisch gevoeliger zijn voor schimmelinfecties. Dit moet in de toekomst verder worden onderzocht. In deze studie hebben we ook een groep van patiënten met een normaal immuunsysteem en grieplongontsteking opgenomen op de intensive care vergeleken met patiënten, ook met een normaal immuunsysteem, voor een gewone longontsteking opgenomen op de intensive care. In de groep met griepvirusinfectie kwam invasieve aspergillose in 15% voor terwijl deze bij andere patiënten, die wel een longontsteking hadden maar geen griep, maar in 5 % voorkomt. Uit deze cijfers concluderen wij dat griep de kans op een *Aspergillus*-infectie gevoelig verhoogt. In **hoofdstuk 10.2** hebben we onderzocht of invasieve aspergillose bij patiënten met griep op de intensive care aanleiding geeft tot een hogere mortaliteit. Bijna de helft van de patiënten met griep en invasieve aspergillose sterft, terwijl 20% van de patiënten met griep zonder *Aspergillus* infectie overlijden als gevolg van deze ernstige ziekte. Met deze studie kunnen we dus concluderen dat invasieve aspergillose een frequente complicatie is bij patiënten opgenomen met grieplongontsteking op de intensive care, die bovendien gepaard gaat met een hoge mortaliteit. Momenteel loopt er een studie waarbij een groep van patiënten met griep op de intensive care antischimmelmedicijn krijgt en een andere groep niet. De studie onderzoekt of het geven van antischimmelmedicijnen het optreden van invasieve aspergillose voorkomt.

TOEKOMSTIG ONDERZOEK

Sinds 15 jaar is het antischimmelmedicijn voriconazole de voorkeursbehandeling voor invasieve aspergillose. Helaas is de mortaliteit van invasieve aspergillose, behandeld met voriconazole nog steeds 25 tot 30%, en dus veel te hoog. Het combineren van 2 antischimmelmedicijnen zou mogelijk kunnen leiden tot een lagere sterfte. Tussen 2008 en 2011 is er een klinische studie verricht om te onderzoeken of het toevoegen van een tweede antischimmelmedicijn aan voriconazole de mortaliteit kan verlagen. Dit bleek het geval, want met combinatietherapie van twee antischimmelmedicijnen was de sterfte 30% lager (19,3%) dan met voriconazole alleen (27,5%). Deze vermindering in mortaliteit van 30% was echter statistisch niet significant, waardoor combinatietherapie in internationale richtlijnen (nog) geen plaats heeft. Een tweede studie is daarom nodig om deze bevinding te bevestigen. Bovendien is er een bijkomende reden om combinatietherapie in Nederland en België te bestuderen: resistentie voor schimmelmedicijnen wordt er in toenemende mate vastgesteld. Dit bemoeilijkt de behandeling van invasieve aspergillose in ernstige mate en gaat gepaard met een hogere sterfte. Zowel van de Belgische als van de Nederlandse overheid hebben we subsidie gekregen om een dergelijke studie uit te voeren. Deze studie zal van start gaan in 2020 en wordt beschreven in **hoofdstuk 11**.

CONCLUSIE

In deze thesis hebben we gepoogd de kennis omtrent invasieve aspergillose te verbeteren met betrekking tot voorkomen, mortaliteit, risicofactoren en diagnose. We hebben aangetoond dat azole-resistente invasieve aspergillose de mortaliteit aanzienlijk verhoogt (**hoofdstuk 3**). De aanpak van azole-resistente invasieve aspergillose is zeer divers in verschillende academische ziekenhuizen (**hoofdstuk 2**). We hebben voor de diagnose en behandeling van (azole-resistente) invasieve aspergillose een uniforme manier van aanpakken geïmplementeerd in alle Nederlandse academische ziekenhuizen en in een aantal Belgische academische ziekenhuizen. Deze Azole-Resitance Management Study (AzoRMan-studie) geeft niet alleen een goed idee van de prevalentie van azole-resistentie, maar we hopen ook dat deze studie de mortaliteit van azole-resistente invasieve aspergillose vermindert. Tussentijdse resultaten van deze AzoRMan-studie zijn beschreven in **hoofdstuk 9** en geven aan dat resistentie wordt gevonden in 8.5% van de patiënten. We beschrijven in **hoofdstuk 4** en **5** verschillende behandelmogelijkheden voor patiënten geïnfecteerd met azole-resistente invasieve aspergillose na een behandeling met liposomaal-amfotericine B. Posaconazole met hoge bloedspiegels of liposomaal-amfotericine B met dosering twee- of driemaal per week zijn een optie

wanneer bepaalde veiligheidsmaatregelen worden in acht genomen. In **hoofdstuk 6**, schrijven we onze ervaring neer met betrekking tot het toedienen van liposomaal-amfotericine B in het hersenkamersysteem of rechtstreeks in het hersenvocht. In **hoofdstuk 7** hebben we aangetoond dat het lateral flow device (LFD) een mooie aanvulling is in het diagnostisch arsenaal voor invasieve aspergillose. In **hoofdstuk 8**, hebben we duidelijk gemaakt dat AsperGenius© PCR een infectie, veroorzaakt door zowel azole-gevoelige als azole-resistente Aspergillus kan detecteren. In **hoofdstuk 10.1** stelden we vast dat een longontsteking veroorzaakt door het griepvirus op de intensive care kan verwikkeld worden door invasieve aspergillose en dit zowel bij patiënten met een normaal als bij patiënten met een onderdrukt immuunsysteem. We tonen aan in **hoofdstuk 10.2** dat deze complicatie gepaard gaat met een sterk verhoogde kans op overlijden. Tot slot, stellen we in **hoofdstuk 11** een nieuwe studie voor die zal starten in 2020 en waarbij het resultaat van de combinatie van twee antischimmelmedicijnen wordt vergeleken met de standaard behandeling van één antischimmelmedicijn. Met deze studie en een aantal actief lopende studies hopen we de uitkomst van patiënten met invasieve aspergillose te verbeteren.

Chapter 14

List of publications

Curriculum vitae

Portfolio

Dankwoord

De stoutste wezels zuipen de beste eieren.
(geïnspireerd door O. Algoet; Nederlands spreekwoord)

LIST OF PUBLICATIONS

This thesis

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CURRICULUM VITAE

Alexander Schauwvlieghe was born on the 22nd of March in Ghent, a beautiful medieval Belgian city. He graduated at the Jesuit College Sint-Barbara in 2005. With the conviction he would like to become a medical doctor, he studied medicine at Ghent University including traineeships at Ospedale Sant'Andrea in Rome and at King's College Hospital in London. Alexander graduated in 2012 with great distinction. Then, he started his Internal Medicine residency at AZ Sint-Jan Hospital in Bruges where he met his wife Judith with whom he is happily married since 2016. Not only Judith stole his heart that year, but Alexander fell in love with two disciplines: Haematology and Infectious Diseases. The second year of his Internal Medicine rotation was performed in Maria Middelares in Ghent. Following two years of Internal Medicine rotation, Alexander started his Haematology fellowship in Ghent University Hospital under the supervision of Prof. dr. F. Offner. During these years, he came in touch with dr. Bart Rijnders from Erasmus University Hospital in Rotterdam at a Boerhaave Infectious Diseases course in Leiden. Bart offered a PhD position on invasive fungal infections and after two years, Alexander left Ghent for a Dutch adventure crossing the Moerdijk in Rotterdam. Alexander combined his PhD work with finishing his Haematology fellowship in Rotterdam, under the supervision of Prof. Dr. J. Cornelissen and dr. P. te Boekhorst. In October 2019, Alexander registered as Internist-Haematologist. Following a 3-year experience in Rotterdam, a 7-month fellowship at University Hospitals Leuven followed. In 2019, he was awarded a place in the Clinical Research Training in Hematology (CRTH) program of the EHA (Eureopean Hematology Association). During his PhD, Alexander helped establishing the Dutch-Belgian Mycoses Study Group, which is an initiative to facilitate collaboration and research on invasive fungal infections by bringing Dutch and Belgian clinicians and researchers together with a common interest in these infections. Alexander is dedicated to continue research improving the care and outcome for patients with hematological conditions that have infectious complications. His future research will focus on de-escalation of antibiotic therapy for febrile neutropenia in patients with longstanding neutropenia and on the diagnosis and management of invasive fungal infections.

DANKWOORD

Het is zover, ik mag eindelijk aan mijn dankwoord beginnen. In oktober 2016 stak ik voor het eerst de Moerdijk over met de wispelturige Beneluxtrein om in een nu wijlen muffe Z-flat van het Erasmus MC zonder enige mogelijkheid tot temperatuurcontrole aan het werk te gaan. Een bokaal met een prachtig uitzicht op de Rotterdamse haven. Het was er lekker knus met mijn Z-flat kamergenoten Anne, Angelique en Lisanne. Tussen al het statistisch gesakker, herschrijven van artikels en beantwoorden van vele e-mails was het een verademing om af en toe een koffie te drinken of een wandeling in "Het Park" te maken. Ik ben mijn kamergenoten dan ook dankbaar om mij als zwervende Belg warm te hebben ontvangen en wegwijs te hebben gemaakt in de voor mij Nieuwe Wereld. Na de verhuis konden we genieten van een nieuw plekje op de 21ste etage met een nog mooier uitzicht.

Hoewel Nederlands en Vlaams als dezelfde taal worden beschouwd, leidde mijn verblijf in Rotterdam al snel tot een babylonische spraakverwarring. Het Vlaamse taalgebruik bleek niet altijd uitwisselbaar met dat van onze noorderburen. In mijn eerste week werd ik bij heel wat mensen op de kamer uitgenodigd. Als Vlaming voelt dit gek aan tenzij enige vrijblijvende promiscuïteit je niet vreemd is. Ook het medische jargon bleek jammer genoeg niet van hetzelfde laken een broek (in Rotterdam: van hetzelfde laken een pak). Toeren werd vervangen door visite lopen en de stap naar een gestructureerd overlegmodel was ook even wennen. Na enige acclimatisatie kon ik gelukkig al enkele dagdagelijkse begrippen zoals hemoculturen, staal, perifeer bloedbeeld en coprocultuur vertalen naar bloedkweken, monster, handdiffje en faeceskweek, respectievelijk. Desondanks bleek communiceren in Nederland niet altijd een eitje. Graag zou ik dan ook mijn Nederlandse collega's willen danken voor hun geduld in alle pogingen om mij te verstaan.

Beste Bart, het is dankzij jou dat mijn carrière een Nederlandse wending heeft gekend. Door het toeval kruisten onze wegen elkaar en mocht ik onder jouw deskundige begeleiding een promotietraject aanvatten. Net als jou werd ik omgedoopt tot Nederbelg met een Brompton. Ik heb onze samenwerking als ontzettend fijn en vlot ervaren. Ik ken niemand die jouw werkijver en efficiëntie kunnen navolgen. Een dag bestaat bij jou werkelijk uit 30 uur. Een multicenter studie lanceren in 5 dagen, 3 miljoen euro binnenrijven voor een grote studie en oh ja, dan ook nog aan de lopende band publiceren en 5 doctorandi begeleiden: je doet het allemaal. Onder het motto 'wie niet waagt, niet wint' zijn we samen aan ons BeNeFit project begonnen, met een bijzonder mooi resultaat tot gevolg. Naast een fantastische mentor heb ik in jou ook een goede vriend gevonden. Ik kijk uit naar de vele grensoverschrijdende gezamenlijke projecten in de toekomst!

Graag wil ik ook mijn opleider (Vlaams: stagemeester), dr. Peter te Boekhorst bedanken, en bij uitbreiding de hele Rotterdamse staf. Ik heb erg veel van jullie manier van geneeskunde doen geleerd! Mijn bijzondere dank gaat ook naar dr. Van Zaanen en dr. Libourel, Henk en Ward, voor de warme ontvangst in het Sint-Franciscus Gasthuis en voor de zelfstandigheid die ik van jullie kreeg. Dank je Pim en Elly, mijn poli-supervisoren (geen gerichte Vlaamse vertaling voorhanden), voor de kennis die ik van jullie kon opdoen! Dank je aan de mede-fellows: Jurjen en Jurriaan voor de fijne overlegmomenten.

Beste professor Cornellissen en professor Verbon, beste Jan en Annelies, dank om mijn promotoren te zijn. Bijna 4 jaar heb ik in het Erasmus MC gewerkt. Ik heb het als zeer verrijkend ervaren en zal uit Rotterdam een grote rugzak kennis en ervaring meenemen voor mijn latere carrière als hematoloog/onderzoeker. Dank je Annelies om bij onze overlegmomenten het overzicht te houden en Jan voor je stimulerende interventies en steun zodat ik aan EHA's Clinical Research Training in Hematology kon deelnemen.

Beste Gentse collega's, ik ben erg blij dat ik ben opgeleid in mijn geboortestad. We hebben een leuke, gezellige dienst en ik ben verheugd jullie collega te worden. Beste professor Offner, beste Fritz, dank dat je mijn atypisch opleidingstraject met Nederlandse escapades altijd hebt ondersteund. Beste professor Kerre, beste Tessa, dank dat je deel wil uitmaken van mijn leescommissie. Beste Ciel, we hebben in het begin van onze opleiding heel wat avondlijke uren doorgebracht en gestreden voor onze patiënten. Je bent een heel goede vriendin geworden waarop ik altijd kan rekenen!

Beste Leuvense collega's, ik ben erg blij dat ik bij jullie zeven maanden heb mogen doorbrengen. Het was tijdens de vreemde COVID-19 periode dat ik te gast was. Beste professor Vandenberghe, beste Peter, dank om mij hartelijk te ontvangen. Beste professor Maertens, beste Johan, het was fijn je droge humor te leren kennen en ik kijk uit naar een verdere productieve samenwerking. Toine, we hebben samen heel wat projecten tot een goed einde gebracht en nog vele projecten lopende. Ik hoop dat we ons inzicht en onze kennis in het domein van infectieziekten bij onze fragiele hematologie patiënten samen, als joint venture, verder kunnen uitdiepen.

Beste Rosanne, dank voor al je inbreng bij het schrijven van het CIA artikel. Je bent nu al een bijna volleerde onderzoeker onder de vleugels van Bart. Beste Albert, het vele werk waar we mee gestart zijn wordt door jou verder gezet. Ik wens je hier veel succes mee.

Beste leden van de leescommissie, hartelijk dank voor de snelle beoordeling van mijn proefschrift. Ook wil ik de leden van de grote commissie bedanken voor jullie deelname aan de oppositie.

Beste Pieter, bro, wat ben ik blij dat mijn Rotterdams avontuur onze band weer nieuw leven heeft ingeblazen. Beiden trekvogels, kruisten onze wegen bij toeval in Rotterdam. We verkenden culinair Rotterdam minstens wekelijks en 's avonds kon ik in jouw flat op mijn matras in de gang blijven overnachten. Je bent een goede en dierbare vriend. Met je heldere en positieve blik help je me te zien wat belangrijk is.

Olivia, dank om mijn vaste treinbuddy te zijn. Het was een eer vaak de grens met jou over te steken.

Beste Olivier, zonder mijn getuige en kameraad had ik nooit de stappen gezet naar een buitenlandse avontuur. Dank om er altijd te zijn voor een goede babbel en een Gulden Draak in de Aba-Jour.

Mama, papa, Pieter-Paul, Leen, Ann-Sofie, Marthe, Emiel en Gust, ook wel de clan Schauwvlieghe. We zijn een hechte familie en delen lief en leed. Het is altijd fijn om een verlengde van mijn thuis te vinden in Antwerpen, Maastricht, Gent of aan zee. Mama, je bent mijn steun en toeverlaat. Je staat altijd paraat om het minste euvel vlot van de baan te helpen. Geen telefoontje is je te veel. Je springt altijd direct op elk uur van de dag om ons bij te staan. Pieter-Paul, de tennis op vrijdagavond met daarna zalig gerstenat is een instituut geworden. Op de vraag wat ik eerst wil/wou hervatten na de lockdown zal het wel dit sportief vrijdags evenement zijn. Papa, dank je voor je luisterbereidheid en interesse in mijn projecten! Je bent altijd paraat om te helpen zoals het nalezen van mijn Nederlandse samenvatting! Lieve moetie, je bent er al een tijdje niet meer. Ik mis onze vele gezellige momenten en babbels. Je bent voor mij een groot voorbeeld en ik weet dat je erg trots moet zijn dat ik nu dit doctoraat tot een goed einde heb gebracht zoals opa! Plus est en vous, ik hoop dat mijn kinderen dit ook trachten na te streven!

Linda, Esther en Jacques, ik ben erg blij in een warme schoonfamilie thuis te zijn. Lieve Linda, geen afstand is jou te ver, geen moeite te groot. Je ovenschotels hebben al menig stad in Vlaanderen bereikt. Dank je om ons te helpen en zo betrokken te zijn. Esther, La Esterella, dank om een toffe meter voor Oscar te zijn en ons te hulp te schieten waar je kan. Jacques, onze sportieve uitjes zijn super ontspannend en dank om als eindredacteur te dienen voor mijn nederlandstalige teksten!

Lieve Judith, mijn darling. Mijn Nederlands avontuur heeft ook heel wat van jou gevraagd. Het was het begin van een nomadenbestaan waarbij we op vele plaatsen gewoond hebben langs de sporen van de Beneluxtrein. Eerst woonden we in Mechelen waarbij je dagelijks naar Leuven moest pendelen. De twee volgende jaren woonden we

in Antwerpen, van waaruit jij ook gedurende een jaar de grens overstak naar Breda, waar je het na een zekere gewenningsperiode best naar je zin had. Het mooiste moest toen nog komen en vol verlangen keken we ernaar uit. Op 24 mei 2019 was hij daar: onze lieve vriend Oscar. Werken is een ding, maar jij en Oscar zijn mijn alles. Elke dag ben ik erg dankbaar voor het lieve gezinnetje waarin ik thuiskom. Zonder jullie was deze promotie nooit een succes geweest.

Dit proefschrift is het slotakkoord geworden van een mooie periode in Rotterdam waar ik ook mijn opleiding heb voltooid. Met gemengde gevoelens van trots, weemoed, voldoening en opluchting zet ik een punt achter dit hoofdstuk.

Het ga jullie allemaal goed,

Zon op al jullie wegen,

Alexander

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student:	Alexander F.A.D. Schauwvlieghe
Departments.	Internal Medicine and Infectious Diseases
	Hematology
Promotors:	Prof. dr. A. Verbon
	Prof. dr. J.J. Cornelissen
Co-promotor:	dr. B. J. A. Rijnders
PhD period:	01.10.2016-31.12.2019

General PhD courses

Basiscursus Regelgeving en Organisatie voor Klinische onderzoekers (BROK-course)	2017	1.5
Open clinica (eCRF course)	2017	0.4
Systematische Literatuuronderzoek in PubMed	2016	0.1
Werken in Endnote	2017	0.1
Scientific Integrity	2019	0.3
E-learning biomedical statistics AMC	2017	1.0
Clinical Research Training in Hematology by EHA	2018-2019	3.0

Specific courses related to internal medicine

Belgian Hematology Society: educational course	2016-2018	2.0
Advanced immunology course	2018	2.0
European Hematology Exam, Stockholm	2018	2.0
International Mycology Course, Nijmegen	2017	1.5

International conferences and courses

58th ASH: Annual Meeting and Exposition, San Diego	2016	2.5
BHS annual meeting, La Hulpe Belgium	2016	1.0
European Hematology Society, Annual meeting, Madrid	2017	2.5
Trends in Medical Mycology, Belgrade, 6-9 October	2017	2.5
59th ASH: Annual Meeting and Exposition, Atlanta	2017	2.5
European Hematology Society, Annual meeting, Stockholm	2018	2.5
60th ASH: Annual Meeting and Exposition, San Diego	2018	2.5
European Hematology Society, annual meeting, Amsterdam	2019	2.5
European Hematology Society, annual meeting, virtual congress	2020	2.5

National presentations

Yearly EMC Internal Medicine days, Antwerp	2017	1.0

Oral presentations and posters:

Oral e-poster presentation, ECCMID, Vienna. Clinical experience with posaconazole high-dose for the treatment of azole-resistant invasive aspergillosis	2018	1.0
Oral presentation at TIMM, Belgrade Influenza-associated aspergillosis: a retrospective cohort study	2017	1.0
Poster presentation at TIMM, Belgrade	2017	1.0
Detection of azole-susceptible and azole-resistant Aspergillus coinfection by PCR	2018	1.0
Presentatie top-publicaties Nederlandse Internisten dagen, Maastricht	2018	1.0
Influenza-associated pulmonary aspergillosis: a retrospective cohort study	2018	
Stopping antibiotic therapy after 72 hours in patients with febrile neutropenia undergoing intesive chemotherapy for AML/MDS (SAFE study): a retrospective compartative cohort study	2020	1.0
Antifungal resistance, does it threaten our patients?	2020	1.0

Teaching activities

Onderwijs infectiologen in opleiding dag 17: infecties na perifere stamceltransplantatie	2017	1.0
Minor Interne Geneeskunde, neutropene koorts	2017	1.0
Optimale toepassing van de antibiotische therapie, influenza en aspergillose, Ede	2018	1.0
Supervision of research internship of medical student drs. R. Verwijs Project: Co-infection influenza and aspergillosis	2017	2.0
Supervision of research internship of medical student drs. S. Algoe Project: Pneumocystis jirovecii infection: avoidable complication?	2018	2.0
Supervision of PhD candidate drs. A. Dunbar	2018	3.0
Project: Invasive fungal infection in the immunocompromised host	2018	2.0

Scholarships and Awards

Young investigator travel award, TIMM 2017	2017
Top publicatie interne geneeskunde Nederland, internistendagen Maastricht	2018

In Vlaanderen kan je patiënten overlopen,
in Nederland niet maar je kan er wel overgaan.