Invasive Aspergillosis in Clinical Hematology

Diagnosis and impact on triazole resistance

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ISBN: 978-94-6361-102-2

Cover design by Ga-Lai Chong
Layout and printing by Optima Grafische Communicatie B.V.

Printing of this thesis was financially supported by the Erasmus University Rotterdam.

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Invasieve aspergillose in klinische hematologie
Diagnostiek en impact van triazole resistentie

Proefschrift

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

Prof. dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

dinsdag 5 juni 2018 om 9:30 uur

door

Ga-Lai Maria Chong
geboren te 's-Gravenhage

Erasmus University Rotterdam

PROMOTIECOMMISSIE

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Voor pappa en mamma

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General introduction and outline of the thesis

INTRODUCTION

Invasive aspergillosis (IA) is a severe opportunistic infection that is mainly caused by *Aspergillus fumigatus* [1,2]. It is the most common invasive fungal disease (IFD) in immunocompromised patients with an underlying hematological disease, including those with acute myeloid leukemia (AML) receiving remission induction or consolidation chemotherapy, and recipients of allogeneic hematopoietic stem cell transplantation (alloHSCT) [1,2]. Not only does it lead to an increase in morbidity and mortality among these patients [3,4], but also to an increase in medical costs [5]. Given these facts, it is essential to optimize management of IA (including early diagnosis, adequate antifungal therapy and prevention) in high-risk patients with an underlying hematological disease.

Currently, the first line therapy is voriconazole, an antifungal agent from the class azoles [6]. Before the introduction of mold-active azoles in the 1990s, mortality rates as high as 90% have been reported in patients with an underlying hematological disease [3,4]. When the recommended therapy with voriconazole is initiated promptly, a relatively low mortality between 26% to 39% at 12 weeks after diagnosis is observed [5,7,8]. Early diagnosis is therefore highly important to treat IA without any delay.

Another strategy in the management of IA is prevention through antifungal prophylaxis. Antifungal prophylaxis with azoles, echinocandins and polyenes have shown to be effective in preventing IA in high-risk patients with an underlying hematological disease [9-12]. At present, mold-active azoles posaconazole and voriconazole are recommended in patients with prolonged neutropenia or in allogeneic alloHSCT recipients with active graft-versus-host disease (GVHD) [6]. Besides reducing the incidence and IA-related mortality, antifungal prophylaxis may also be cost-effective. This thesis focuses on IA in high-risk patients with an underlying hematological disease and in particular on its epidemiology, prevention and diagnostics.

INVASIVE ASPERGILLOSIS: INCIDENCE, MORTALITY, RISK FACTORS AND PREVENTION

In the management of IA, it is important to monitor the local incidence and mortality in high-risk patients as these are subjected to continuous changes, such as alterations in antifungal prophylaxis and the emergence of azole-resistant *A. fumigatus* and intrinsically azole-resistant sibling species. Moreover, risk factors should be identified to recognize the subset of patients who are at highest risk and will benefit most from antifungal prophylaxis. **Chapter 2** describes the incidence, mortality and risk factors of IA in alloHSCT recipients who were transplanted in the Erasmus University Medical Center over the past decade.

As mentioned earlier, IA is not only associated with an increased morbidity and mortality, but also with a substantially increase of medical costs in patients with an underlying hematological disease [3-5,7,8]. Given these observations and the fact that hospital resources are not infinite, preventing IA is an attractive strategy to reduce IA-related mortality and costs. Although posaconazole and voriconazole are recommended as antifungal prophylaxis [6], other antifungals from other classes can be used as prophylaxis as well [10,12]. Inhalation of aerosolized liposomal amphotericin-B (L-AmB) has been shown to be effective and safe in preventing IA [10,13]. It has the advantages of (i) no systemic side effects in compare to intravenous L-AmB and other antifungal prophylaxis, and (ii) retaining azoles in case of therapy. In 2008, it was implemented as standard of care for patients with AML receiving intensive chemotherapy in the Erasmus University Medical Center. **Chapter 3** describes the efficacy and cost-effectiveness of L-AmB inhalations on the incidence and mortality of IA in high-risk patients with AML.

DIAGNOSIS OF CEREBRAL ASPERGILLOSIS

The diagnosis of IA is made according to the revised definitions of the European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG) [14]. In the revised definitions, one of the microbiological criteria is the detection of galactomannan (GM), a cell-wall component of *Aspergillus* that is released during cell growth [15]. Although the detection of GM antigen has been well validated in serum and bronchoalveolar lavage (BAL) fluid of immunocompromised patients [16,17], less is known about its diagnostic performance in cerebrospinal fluid (CSF) to diagnose cerebral aspergillosis (CA). Based on three small studies, GM antigen testing in CSF seems to be promising, even though no formal cut-off was established [18-20]. **Chapter 4** describes the results of a study that investigated the diagnostic performance of GM antigen in CSF of patients with suspected CA.

DETECTION OF AZOLE-RESISTANT *ASPERGILLUS FUMIGATUS* IN INVASIVE ASPERGILLOSIS

Over the past decade, azole resistance in *A. fumigatus* has emerged worldwide [21,22]. This is worrisome as IA with an azole-resistant *A. fumigatus* is associated with very high mortality rates of almost 90% [23,24]. Azole resistance is often 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, e.g. TR_{34}/L98H and TR_{46}/T289A/Y121F, account for a large part of azole-resistant mechanisms [23,25,26] and are

believed to have developed in the environment due to azole exposure in agriculture [27,28].

Detection of azole resistance in IA is challenging for two reasons. First, a positive culture is required for conventional detection of resistance. However, cultures are positive in at most one-quarter of the cases and therefore diagnosis is often made indirectly by detection of galactomannan (GM) antigen [8,29]. Second, conventional microbiological tools for azole susceptibility testing are not widely available and time consuming. The lack of a fast and readily available azole susceptibility testing compromises the initiation of adequate therapy in case of azole resistance. Hence, there is a need for development of newer diagnostic techniques to intercept this problem.

Among the novel diagnostics are *Aspergillus* polymerase chain reaction (PCR) assays that detect and identify *Aspergillus* to the species level in different specimens [30-32]. The AsperGenius® multiplex real-time PCR is a commercially available *Aspergillus* assay. In addition to detecting *Aspergillus*, it detects the aforementioned two most common mutation combinations of *A. fumigatus* that are associated with azole resistance. Chapter 5.1 describes the results of the single-center study that investigated the diagnostic performance of this assay on BAL fluid samples of patients from the hematology department and intensive care unit. To confirm the results of the single-center study, subsequently a multicenter study was performed in a larger BAL sample set from patients with an underlying hematological disease. Moreover, we evaluated in this second study if PCR detection of the aforementioned mutation combinations correlated with azole treatment failure and mortality. The results of this multicenter study are described in Chapter 5.2.

DETECTION OF AZOLE-RESISTANT SIBLING SPECIES IN INVASIVE ASPERGILLOSIS

IA is mainly caused by *Aspergillus fumigatus*, an *Aspergillus* species from the section *Fumigati* [1,2]. Occasionally, IA is caused by *Aspergillus* species that are morphologically similar to *A. fumigatus* [33,34]. These so called 'sibling species' or 'cryptic species' also belong to the *Aspergillus* section *Fumigati* and are often intrinsically resistant to azoles. As voriconazole is the recommended first line therapy [6], fast identification and susceptibility testing of these sibling species is important to select the appropriate antifungal therapy. However, this is problematic as cultures are not available in the majority of the patients with IA [8,29]. Moreover, if a culture is available, identification and susceptibility testing is difficult as these sibling species often have poor sporulation and can only be distinguished by additional testing. **Chapter 6** describes two patients with proven IA

caused by azole-resistant sibling species and the contribution of the aforementioned AsperGenius® assay in their identification.

SUMMARY

Several studies were performed to investigate the incidence, mortality, risk factors and diagnostics of IA. **Chapter 2** focusses on the incidence, mortality and risk factors of IA in alloHSCT recipients. **Chapter 3** describes the efficacy and cost-effectiveness of L-AmB inhalations on the incidence and mortality of IA in patients with AML receiving intensive chemotherapy. The subsequent chapters focus on the diagnostics in IA. In **chapter 4**, the diagnostic performance of GM in CSF is described. **Chapter 5.1 and 5.1** present the results of two studies that evaluated the diagnostic performance of a novel PCR-assay that can not only detect *Aspergillus*, but also the two most common *Cyp51A* mutations in *A. fumigatus* that confer to azole resistance. **Chapter 6** describes the contribution of this novel PCR-assay in identifying sibling species and is followed by a general discussion in **chapter 7**.

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Chapter 2

Incidence, outcome and risk factors for invasive aspergillosis in 663 allogeneic hematopoietic stem cell transplantation recipients. A nested case control study.

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ABSTRACT

Introduction

Allogeneic hematopoietic stem cell transplantation (alloHSCT) recipients are at risk for invasive aspergillosis (IA). We conducted a retrospective study to determine incidence, outcome and risk factors for IA in alloHSCT recipients.

Methods

Patients who underwent their first alloHSCT between 2004 and 2014, were included. IA was classified according to the proven or probable EORTC/MSG criteria. In addition, patients with a positive microbiological criterium but with non-specific radiological findings, were defined as having non-classifiable IA. The 12-month IA incidence and mortality were determined. Within the total cohort, a nested case-control study was performed to determine risk factors for IA.

Results

Of 663 alloHSCT recipients, 86 developed IA within the 12 months post-transplantation (1.8% proven, 7.4% probable and 3.8% non-classifiable IA). Twelve-month all-cause mortality was 45.6% in patients with IA and 26.2% in those without IA (p-value<0.01). For the nested case-control study, 99 patients with proven/probable/non-classifiable IA were compared to 198 patients without IA. Backward logistic regression showed the following independent risk factors: neutropenia (OR 16.22; p-value<0.01), lymphocytopenia (OR 5.70; p-value<0.01), reactivation of cytomegalovirus (OR 5.27; p-value=0.02), creatinine >135 µmol/l (OR 2.48; p-value=0.03), pulmonary comorbidity (OR 2.67; p-value=0.01); and use of prednisolone 1-1500 milligram (OR 4.06; p-value<0.01) and >1500 milligram (OR 45.05; p<0.01) in the 4 weeks preceding the IA. Pre-transplant IA was not identified as an independent risk factor for post-transplant IA.

Conclusion

At twelve months post-transplantation, 13.0% of the patients had developed IA and had a decreased survival compared to patients without IA.. Several independent risk factors were found, but surprisingly pre-transplant IA was not among them. A higher dose of prednisolone was associated with a higher risk for IA, suggesting a dose-effect relationship.

INTRODUCTION

Invasive aspergillosis (IA) is a common invasive fungal disease (IFD) in recipients of allogeneic hematopoietic stem cell transplantation (alloHSCT) [1-12]. The reported incidences vary between 2.3% and 15.0%, and mortality is substantial in this particular patient group [1-4,6-8,10]. Primary prophylaxis with voriconazole or posaconazole can reduce the incidence of IA in alloHSCT recipients and is recommended in those with graft-versus-host disease (GVHD) who are in need of systemic corticosteroid or other anti-GVHD therapies [13-15]. However, apart from GVHD, many other risk factors for post-transplant IA have been described, such as older age, neutropenia, non-related donor, reactivation of cytomegalovirus (CMV) and a history of pre-transplant IFD [1-4,6,9,10,12,16,17]. Also, a genetic predisposition has been identified [18]. IA is most frequently observed in the first year post-transplantation, but a substantial part of the recipients develops IA later [1,6,7]. Therefore, the best timing and duration of primary prophylaxis should be individualized. Recognizing risk factors may help in selecting those patients that are at highest risk and benefit most of primary prophylaxis. We conducted a retrospective single-center study to study the incidence, mortality and risk factors for post-transplant IA in alloHSCT recipients.

METHODS

Study design

The study was performed at Erasmus University Medical Center (Rotterdam, the Netherlands), a tertiary referral hospital with three hematology departments at two sites. We reviewed medical files of all patients who underwent their first alloHSCT between January 2004 and December 2014. Data were obtained till the end of the study (1st of April, 2015). The following information was retrieved from medical files: age, sex, underlying hematological disease, date and donor type of alloHSCT, conditioning regimen pre-transplantation, relapse of hematological disease post-alloHSCT, documentation of IFD before and after alloHSCT, acute and chronic GVHD, renal and hepatic impairment, pulmonary comorbidity, reactivation of CMV, neutropenia, lymphocytopenia, number of CD3+ T cells, use of systemic corticosteroids, European Group for Blood and Marrow Transplantation (EBMT) risk score and mortality.

The study consisted of two parts. First, in the total cohort we assessed (i) the incidence of IA at 12 months post-transplantation and overall, and (ii) the incidence of IA per calendar year of transplantation and per donor type. Also, we determined (i) the all-cause mortality at 12 months post-transplantation and overall, (ii) the IA-related mortality, defined as mortality within 6 weeks of proven, probable or non-classifiable

IA diagnosis, and (iii) the non-relapse mortality (NRM). Furthermore, the incidence and timing of IFD other than IA were evaluated.

Second, a nested case-control study was performed within the total cohort to identify risk factors for developing post-transplant IA. Cases were defined as patients with proven, probable or non-classifiable IA after alloHSCT. Controls were patients without IA. The following patients were excluded: (i) patients with possible IA, (ii) patients with IFD other than IA, (iii) patients having an episode of IA before alloHSCT who continued to receive on antifungal therapy for this episode after alloHSCT. These exclusion criteria were used to ascertain as much as possible that the cases had and the controls had not developed an IA after alloHSCT. Every case was matched randomly to 2 controls using age at transplant (±5 years), year of transplant (±1 year) and follow-up time as matching variables. Matching was performed using IBM® SPSS, version 21 (plug-in "Fuzzy"). To ensure that risk factors could also be studied in controls, the follow-up time of the two matched controls was at least as long as the case. Except for lymphocytopenia and CD3+ T cell, variables that varied over time (i.e. acute or chronic GVHD, renal or hepatic impairment, reactivation of CMV, neutropenia, use of prednisolone) were assessed during the time frame of 1 month preceding the diagnosis of IA in the cases. In the rare event that renal and hepatic function had not been tested in the indicated time period, the last observation was carried forward with a maximum of 6 months. For controls, the same variables were obtained at the number of days after alloHSCT that corresponded to this time-frame in the corresponding matched case. Lymphocytopenia and CD3+ T cells were assessed 3 months or as the first encountered number preceding IA diagnosis, respectively, since these 2 variables were not determined in time frame of 1 month in the majority of the patients.

Antifungal prophylaxis with fluconazole 400 milligrams daily was given during the post-transplantation conditioning-induced neutropenia. Primary prophylaxis for IA consisted of voriconazole 200 milligrams twice daily and was prescribed to patients with GVHD who were treated with second line anti-GVHD treatment. First line treatment consisted of voriconazole in case patients were not treated with primary prophylaxis beforehand.

Definition of IFD

IFD was categorized as proven, probable or possible according to the revised European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) consensus criteria [19]. In addition, patients with appropriate host criteria and positive microbiological findings but with non-specific radiological features were classified as *non-classifiable* disease. Although this category is not yet included in the EORTC/MSG definitions, these patients are treated similarly to those with probable IA given their similar outcome [20]. The same diagnostic protocol, which has

been described elsewhere [21], was used in the entire time period. If multiple periods of IA were suspected for a given patient, the period with the highest IA classification was selected.

Definitions of potential risk factors

To assess renal and hepatic impairment as risk factors for IA, the highest levels of creatinine, bilirubin and alanine aminotransferase (ALAT) in the 1-month observation period were used. To grade hepatic impairment, we used and modified the Common Terminology Criteria for Adverse Events (version 4) of the National Cancer Institute [22]. Hepatic impairment was graded after the highest grade for total bilirubin or ALAT level. Pulmonary comorbidity was based on the pulmonary function testing that was performed as a standard procedure prior to alloHSCT and categorized according to the Hematopoietic Cell Transplantation-Comorbidity Index (HCT-CI) [23]. Neutropenia was defined as a neutrophil count of $<0.5x10^9$/l for 10 consecutive days or more and lymphocytopenia as a lymphocyte count of $<1.0x10^9$/l. Acute and chronic GVHD were defined according to the updated Glucksberg classification and National Institutes of Health Consensus, respectively [24,25]. All patients were categorized according to the EBMT risk score [26]. For the use of corticosteroids, the cumulative dose in milligrams of prednisolone was calculated in the indicated time frame.

Statistical analysis

Statistical analysis was performed using IBM® SPSS (version 21) and Stata (version 13). Clinical characteristics were analyzed using Chi-square test or Mann-Whitney U test as appropriate. We used a backward likelihood logistic regression to determine potential risk factors for IA. A p-value of <0.05 was considered statistically significant.

RESULTS

In total, 663 patients received their first alloHSCT between January 2004 and December 2014. The median age at alloHSCT was 52.8 years (range 16.7 to 71.1). The most frequent underlying hematological disease was acute leukemia (53.5%), followed by lymphoma (11.9%), myelodysplastic syndrome (9.2%), myeloproliferative neoplasms (8.6%), plasma cell neoplasm (6.9%), chronic lymphocytic leukemia (4.7%) and other hematological disorders (5.1%). The mean follow-up was 39.8 months (range 0 to 134.5).

Proven, probable, possible or non-classifiable IA was found in 109 (16.4%) recipients after 12 months post-alloHSCT and overall in 137 (20.7%)(table 1). Over a period of 10 years, the 12-month incidences of IA ranged from 6.4% to 33.3% per alloHSCT year ($p<0.01$)(table 2). An increase in the incidence was observed in 2012 and 2013 (figure

1). The 12-month incidences in matched related, matched unrelated and cord blood alloHSCT were 11.8%, 17.4% and 27.3% (p<0.01), respectively. The mean time from alloHSCT to development of IA was 266 days with a range of 9 to 2093 days (figure 2). In the total cohort, the all-cause mortality 12 months after alloHSCT was 29.1% and the overall all-cause mortality was 45.2%. Patients with proven, probable and non-classifiable IA had a higher mortality in compared to patients without IA at 12 months (45.6% versus 26.2%; p-value<0.001) as well as in overall (63.1% versus 41.1%; p-value<0.001). The IA-related mortality was 26.3% (36/137). Figure 3 shows the cumulative incidence of NRM with relapse as competing risk of patients with IA versus those without IA (NRM at 5 years 49±5% versus 16±2%, respectively, p<0.001).

Table 1. Invasive aspergillosis (IA) after allogeneic hematopoietic stem cell transplantation (alloHSCT) per donor type.

Classification of IA	Number of patients (%) at 12 months				Number of patients (%) in entire follow-up time or end of the study			
	Total n = 663	Matched related n = 270	Matched unrelated n = 305	Cord blood n = 88	Total n = 663	Matched related n = 270	Matched unrelated n = 305	Cord blood n = 88
IA (all)	109 (16.4)	32 (11.9)	53 (17.4)	24 (27.3)	137 (20.7)	43 (15.9)	68 (22.3)	26 (29.5)
Proven	12 (1.8)	2 (0.7)	5 (1.6)	5 (5.7)	13 (2.0)	2 (0.7)	6 (2.0)	5 (5.7)
Probable	49 (7.4)	12 (4.4)	29 (9.5)	8 (9.1)	58 (8.7)	15 (5.6)	34 (11.1)	9 (10.2)
Possible	23 (3.5)	11 (4.1)	6 (2.0)	6 (6.8)	34 (5.1)	15 (5.6)	12 (3.9)	7 (8.0)
Non-classifiable	25 (3.8)	7 (2.6)	13 (4.3)	5 (5.7)	32 (4.8)	11 (4.1)	16 (5.2)	5 (5.7)
No IA	554 (83.6)	238 (88.1)	252 (82.6)	64 (72.7)	526 (79.3)	227 (84.1)	237 (77.7)	62 (70.5)

Table 2. Invasive aspergillosis (IA) after allogeneic hematopoietic stem cell transplantation (alloHSCT) per transplantation year.

Classification of IA	Number of patients (%) per year of alloHSCT									
	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
IA										
Possible	0	0	3 (5.1)	1 (1.8)	1 (1.9)	0	2 (4.2)	3 (4.2)	3 (4.9)	8 (10.7)
Probable	6 (9.8)	1 (2.1)	1 (1.7)	3 (5.4)	1 (1.9)	2 (2.9)	4 (8.3)	5 (7.0)	6 (9.8)	13 (17.3)
Proven	2 (3.3)	0	1 (1.7)	0	1 (1.9)	3 (4.4)	0	2 (2.8)	2 (3.3)	0
Non-classifiable	1 (1.6)	2 (4.3)	1 (1.7)	4 (7.1)	1 (1.9)	2 (2.9)	1 (2.1)	2 (2.8)	3 (4.9)	4 (5.3)
No	52 (85.2)	44 (93.6)	53 (89.8)	48 (85.7)	50 (92.6)	61 (89.7)	41 (85.4)	59 (83.1)	47 (77.0)	50 (66.7)

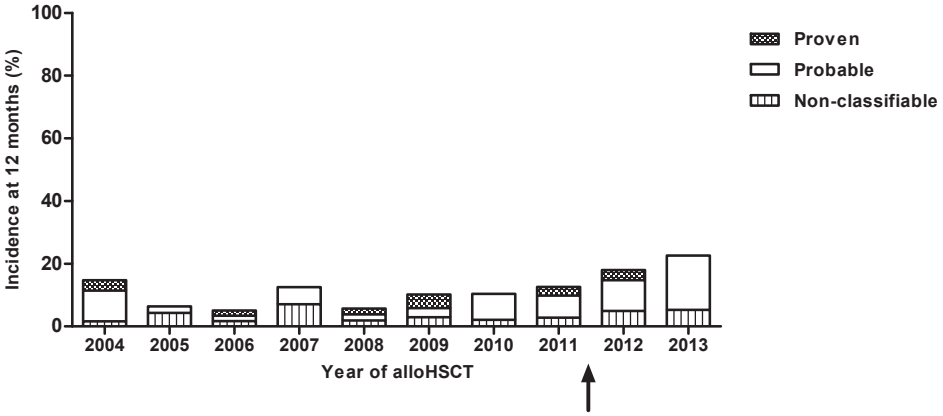

Figure 1. Incidence in percentage of invasive aspergillosis at 12 months per year of allogeneic hematopoietic stem cell transplantation. Arrow: In mid-2011, a long-lasting demolition and construction of a neighboring hospital building started at the site where patients underwent their allogeneic hematopoietic stem cell transplantation.

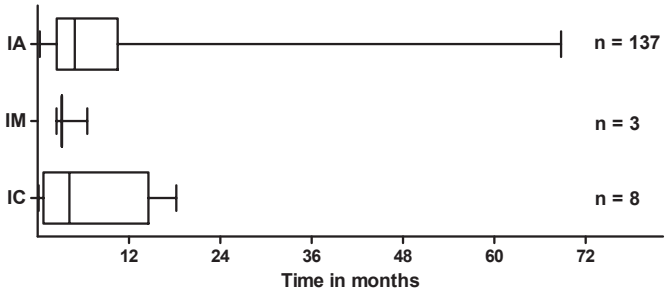

Figure 2. Time distribution from allogeneic hematopoietic stem cell transplantation to diagnosis of invasive aspergillosis (IA), invasive mucormycosis (IM) and invasive candidiasis (IC).

Aspergillus cultures were positive in 59 of the 103 patients with proven, probable or non-classifiable IA. Three cultures were isolated from lung biopsies, 1 from sinus biopsy, 1 from cerebral abscess and the remaining from respiratory specimens (sputum and/or bronchoalveolar lavage). *A. fumigatus* was found most commonly (n=53), followed by *A. terreus* (n=4), *A. flavus* (n=2) and *A. niger* (n=1). Two patients had a co-infection with an *A. fumigatus* and *A terreus.* Cultures with azole-resistant *A. fumigatus* were detected in 6 patients with proven (n=1), probable (n=3) and non-classifiable IA (n=2), and were isolated in the period of 2011 to 2014. Two of the 6 (33%) patients infected with an azole-resistant *A. fumigatus* died within 6 weeks after diagnosis.

IFD other than IA was found in 13 patients, of whom 11 within 12-months post-transplantation. Eight patients developed invasive candidiasis (IC) due to *C. albicans* (n=4), *C. glabrata* (n=2), *C. tropicalis* (n=1) and *C. species* (n=2). One patient had a co-infection

with *C. albicans* and *C. glabrata*. Invasive mucormycosis was observed in 3 patients (all 3 *rhizomucor* species). Two patients were diagnosed with proven IFD based on a positive biopsy, but no species was determined. Nine of these 13 patients were also diagnosed with IA, of whom 5 concurrently with the other IFD.

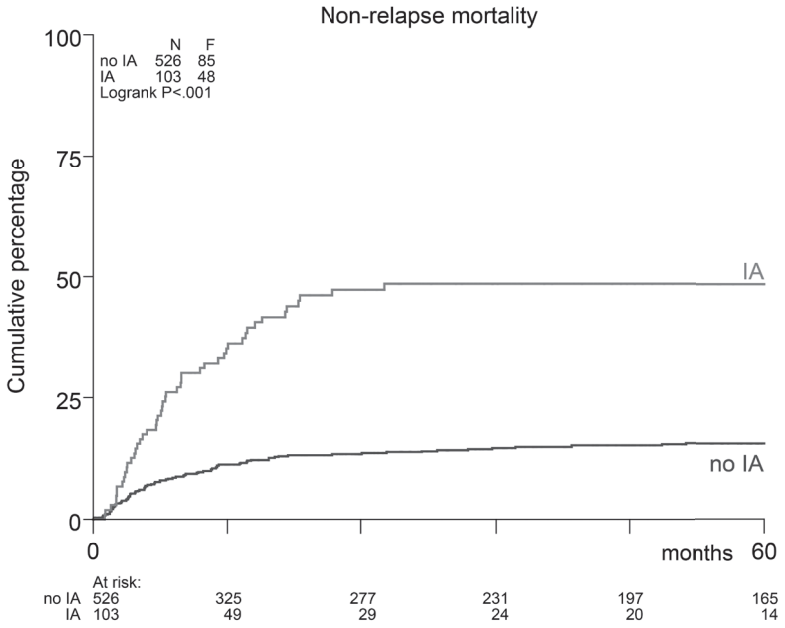

Figure 3. Non-relapse mortality in patients with proven, probable and non-classifiable invasive aspergillosis (IA) versus patients without IA.

Within the total cohort, a nested case-control study was performed to determine the risk factors for IA. Figure 4 shows the inclusion for the nested case-control study. Thirty-nine patients were excluded: 34 patients with possible IA, 4 with IFD other than IA, 1 who was already diagnosed with an IA before alloHSCT. Three more patients were excluded because of an uncertain diagnosis of IA as they recovered without antifungal treatment. Therefore, 103 potential cases and 518 potential controls were available. Matching yielded no controls for 4 cases. Consequently, 99 patients with IA (13 proven, 55 probable and 31 non-classifiable) were matched to 198 controls according to age, year of alloHSCT and follow-up duration. The clinical characteristics of the nested case-control study are shown in table 3. Cases suffered more often from renal and liver impairment, reactivation of CMV and neutropenia during the month preceding IA. Furthermore, acute or chronic GVHD and the use of systemic corticosteroids was significantly higher in the group with IA. After performing a backward logistic regression, the following risk

factors were found: neutropenia, lymphocytopenia, pulmonary comorbidity, reactivation of CMV, creatinine >135 µmol/l and use of corticosteroids (table 4). A higher cumulative dose of corticosteroids in the month preceding IA was associated with a higher risk for IA. Pre-transplant IA, acute and chronic GVHD were eliminated among others in the analysis. Patients receiving corticosteroid therapy and those with acute or chronic GVHD are a large overlapping population. Therefore, an extra analysis was performed to investigate the role of GVHD when acute and chronic GVHD were taken in one variable. Univariate analysis showed that the presence of any GVHD (acute GVHD grade II-IV or chronic moderate/severe GVHD) was associated with an odds ratio of 4.03 (95% CI 2.38 – 6.81; p<0.001). However, when any GVHD was added to the existing model, it was again eliminated (OR 0.83, 95% CI 0.33 – 2.11; p=0.70), and the same risk factors as described above remained significant.

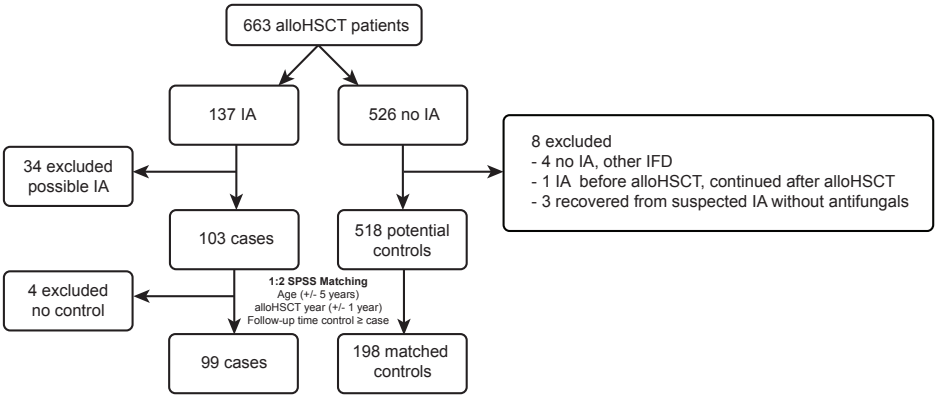

Figure 4. Inclusion for azole therapy failure and 6-week mortality analysis.
AlloHSCT, allogeneic hematopoietic stem cell transplantation. IA, invasive aspergillosis. IFD, invasive fungal disease.

Table 3. Clinical characteristics of 297 allogeneic hematopoietic stem cell transplantation (alloHSCT) recipients included in the nested case-control study.

	Patients without IA[a] n = 198	Patients with proven, probable or non-classifiable IA[b] n = 99	p-value
Age at alloHSCT in years, median (range)	54.9 (20 – 70)	54.0 (21 – 71)	0.889
Male sex, n (%)	110 (55.6)	59 (59.6)	0.507
Underlying disease, n (%)			0.315
Acute leukemia	102 (51.5)	42 (42.4)	
Lymphoma	26 (13.1)	14 (14.1)	
Other	70 (35.4)	43 (43.4)	
Donor type, n (%)			0.071
Matched related donor	76 (38.4)	26 (26.3)	
Matched unrelated donor	99 (50.0)	55 (55.6)	
Cord blood	23 (11.6)	18 (18.2)	
Myeloablative conditioning, n (%)	33 (16.7)	17 (17.2)	0.883
Use of anti-thymocyte globulin during conditioning, n (%)	45 (22.7)	22 (22.2)	0.922
Proven, probable, non-classifiable or possible IA before alloHSCT, n (%)	28 (14.1)	14 (14.1)	1.000
Serum creatinine >135 umol/l, n (%)	38 (19.2)	37 (37.4)	0.001
Hepatic impairment, n (%)			<0.001
ALAT and bilirubine < upper normal limit	66 (33.3)	8 (8.1)	
ALAT and/or bilirubine grade I	73 (36.9)	30 (30.3)	0.262
ALAT and/or bilirubine grade II	33 (16.7)	23 (23.2)	0.173
ALAT and/or bilirubine grade III - IV	26 (13.1)	38 (38.4)	<0.001
Moderate or severe pulmonary comorbidity, n (%)	69 (34.8)	49 (49.5)	0.015
CMV reactivation, n (%)	5 (2.5)	21 (21.2)	<0.001
Neutropenia of ≥10 days, n (%)	11 (5.6)	25 (25.3)	<0.001
Lymphocytopenia, n (%)	118 (60.2)	90 (91.8)	<0.001
CD3+ T cells, median (range)	0.442 (0.005 - 5.319)	0.337 (0.000 - 2.218)	0.006
Acute GVHD, n (%)			<0.001
No acute GVHD or acute GVHD grade I	181 (91.4)	72 (72.7)	
Grade II - IV	17 (8.6)	27 (27.3)	
Chronic GVHD, n (%)			0.009
No chronic GVHD or limited chronic GVHD	175 (88.4)	76 (76.8)	
Moderate or extensive	23 (11.6)	23 (23.2)	
Use of corticosteroids (prednisolone equivalent dose in milligrams), n (%)			<0.001
0	127 (64.1)	21 (21.2)	
1 to 1500	59 (29.8)	34 (34.3)	0.393
>1500	12 (6.1)	43 (43.4)	<0.001
Insufficient data	0	1 (1.0)	-

Table 3. Clinical characteristics of 297 allogeneic hematopoietic stem cell transplantation (alloHSCT) recipients included in the nested case-control study. (*continued*)

	Patients without IA[a] n = 198	Patients with proven, probable or non-classifiable IA[b] n = 99	p-value
EBMT risk score, n (%)			0.122
0 to 2	52 (26.3)	18 (18.2)	
3 to 7	146 (73.7)	81 (81.8)	

[a] No IA was defined as no proven IA, no probable IA, no non-classifiable IA or no possible invasive fungal disease.

[b] IA, invasive aspergillosis. Proven and probable IA was defined according to the revised European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) criteria. Non-classifiable is defined as a patient with EORTC/MSG host and microbiological criteria fulfilled and a pulmonary infiltrate without a halo or air-crescent or well-defined nodule.

Table 4. Risk factors for developing invasive aspergillosis after allogeneic hematopoietic stem cell transplantation according to backward likelihood logistic regression analysis.

Variable	Odds ratio (95% CI)	p-value
Neutropenia	16.26 (5.64 - 46.71)	0.000
Lymphocytopenia	5.70 (1.88 - 17.31)	0.002
Reactivation of cytomegalovirus	5.27 (1.36 - 20.36)	0.016
Creatinine >135 µmol/l	2.48 (1.10 - 5.57)	0.028
Moderate / severe pulmonary comorbiditiy	2.67 (1.22 - 5.85)	0.014
Use of prednisolone		
1 to 1500 milligrams	4.06 (1.62 - 10.16)	0.003
>1500 milligrams	45.05 (14.35 - 141.42)	0.000

DISCUSSION

In this retrospective study of 663 alloHSCT recipients, we found an incidence of proven, probable, non-classifiable IA of 13.0% within the 12-months after transplantation and 15.5% in the entire follow-up time available. As expected, all-cause mortality was significantly higher in patients with than without IA. Moreover, the independent risk factors for IA we observed were neutropenia, lymphocytopenia, pulmonary comorbidity, CMV reactivation, renal impairment and the use of systemic corticosteroids. A higher corticosteroid use was associated with a higher risk of IA.

The incidence, mortality and risk factors of IA were investigated in recipients who received their alloHSCT in our hospital over a decade. Only the period with the highest IA classification per patient was registered. Therefore, the true incidence of IA may be underestimated as some patients were treated with antifungal therapy more than once. Previous studies reported mostly on the incidences of proven and probable IA. In the

current study, we found a 12-month and overall incidence of proven and probable IA of 9.2% and 10.7% respectively. These observed incidences fall within the previously reported incidences of 12-month (2.3% to 7.4%) and overall (2.5% to 15%) risk respectively [1-4,6-8,10]. A significant increased incidence was found among recipients of alternative donor transplants. This observation was also seen in other studies [3,5,8]. However, we did not find donor type to be an independent risk factor for developing IA in the nested case-control study. This may have to do with the smaller sample size of the nested case-control group. Another interesting finding was that 20.4% of all the infections occurred after the first year of transplantation, indicating a continued risk in certain alloHSCT recipients, which has been previously described as well [1,6,11].

In 2012 and 2013, there was a significant increase in the incidence of IA. There may be different reasons for this observation. First, a long-lasting demolition and renovation of a neighboring hospital building was initiated from 2011 onwards at one of the two sites where all alloHSCT patients were transplanted and followed after alloHSCT. Hospital constructions or demolitions have been associated with increased IA in patients with underlying hematological disease [27,28]. Second, azole resistance has emerged over the past decade [29]. A recent study found 16.2% resistance against voriconazole in cultures of *A. fumigatus* that were isolated between 2011 to 2013 from high-risk patients of a Dutch tertiary referral hospital [30]. In our total cohort, 6 recipients had probable or non-classifiable IA due to azole-resistant *A. fumigatus*. As fungal cultures often remain negative and because antifungal susceptibility testing was not a standard procedure in the first half of the observation period, the actual number of patients infected with azole-resistant *A. fumigatus* may have been higher. As primary prophylaxis with triazoles will not prevent azole-resistant IA, the emergence of azole resistance may have contributed to the increased incidence in IA as well. Formerly, primary prophylaxis was prescribed in our center to alloHSCT recipients with GVHD who were treated with second line anti-GVHD treatment. More recently, it was extended to patients who are treated with high-dose systemic corticosteroid therapy.

Patients with proven, probable or non-classifiable IA had a significantly higher mortality compared to patients without IA in the total cohort. In the total cohort, we found an IA-related mortality (=6-week mortality after diagnosis of IA) of 26.3%. Two other studies have described the mortality 6 weeks after diagnosis of IA: Neofytos et al. found a comparable 6-week mortality of 21.5%, but the 6-week mortality observed by Nucci et al. was substantially higher (63%) [8,11]. However, the population studied by these authors has been a more heterogeneous group of patients, including recipients of autologous hematopoietic stem cell transplantation.

To investigate potential risk factors for IA, we performed a nested case-control study within the total patient cohort. Except for lymphocytopenia and CD3+ T cells, all variables that changed over time were assessed in the month before diagnosis of IA in cases and

in the same time-frame post-alloHSCT in the controls. This design was chosen to ensure that the identified time-varying risk factors really contributed to IA. It is similar to the study design of Corzo-León et al. [1], except that our cases also consisted of recipients with non-classifiable IA in addition to those with proven and probable IA. We think it is correct to include non-classifiable IA cases as well because they have the same outcome and are treated in the same way as patients with probable or proven IA [20]. Neutropenia, lymphocytopenia, pulmonary comorbidity, reactivation of CMV, renal impairment and use of prednisolone were found to be independent risk factors. Moreover, the use of a higher dose of prednisolone was associated with a higher risk for developing IA, suggesting that the effect of prednisolone on IA is dose dependent. Neutropenia, CMV reactivation and corticosteroid use have been previously described as important risk factors for IFD [2,6,12,16,17]. However, this study is the first to identify renal impairment as an independent risk factor for IA [1,11,16]. Remarkably, and in contrast to some of the other studies, the diagnoses of pre-transplantation IA or acute/chronic GVHD were not found to be independent risk factors [1-3,6,9,10,12,16]. Perhaps pre-transplant IA was not found to be risk factor, because patients with pre-transplant IA are often treated with secondary prophylaxis during alloHSCT. Also, selection bias may partially explain this finding because a subset of patients in need for an alloHSCT with uncontrolled IA may no longer be eligible for the transplantation. GVHD was analyzed as acute and chronic GVHD and in an additional analysis as one single variable, but was not found to be a risk factor. As patients with acute or chronic GVHD are almost always treated with corticosteroids, these variables are strongly associated. GVHD did not remain significant in the final model presumably because of the stronger effects of corticosteroids. The risk further increased when the cumulative dose of corticosteroids increased.

The major limitation of this study was its single-center and retrospective design. Therefore, the analysis of potential risk factors was limited to the available variables and population size. Ideally, a multi-center prospective study should be performed to determine the incidence, mortality, risk factors and the impact of primary prophylaxis. Especially the efficacy of primary prophylaxis should be evaluated in due time in the light of increasing azole resistance. However, this would have its own challenges as the policies of primary prophylaxis after alloHSCT differ among the Dutch transplantation hospitals and may change if the incidence of azole resistance continues to increase over the coming years.

In conclusion, an incidence of proven, probable and non-classifiable IA was found of 13.0% at 12-months post-transplantation and 15.5% in overall. Mortality was significantly higher in patients with IA than those without. Different independent risk factors were found: neutropenia, lymphocytopenia, pulmonary comorbidity, reactivation of CMV, renal impairment and use of prednisolone. The results are important as it may help

clinicians in starting or adjusting antifungal prophylaxis in the appropriate patient at the correct time.

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Chapter 3

Aerosolized liposomal amphotericin-B to prevent aspergillosis in acute myeloid leukemia: Efficacy and cost-effectiveness in real-life

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Int J Antimicrob Agents 2015;46:82-87

ABSTRACT

Background

Chemotherapy-induced neutropenia can be complicated by invasive pulmonary aspergillosis (IPA). In 2008, liposomal amphotericin-B (L-AmB) inhalation was shown to prevent IPA in a placebo-controlled trial. Patients with acute myeloid leukemia (AML) are the subset of hematology patients at high risk for IPA. In 2008, L-AmB inhalation prophylaxis became standard of care for all AML patients in Erasmus University Medical Center. In this study, the efficacy and cost-effectiveness of L-AmB inhalation were evaluated in a prospective cohort of AML patients.

Methods

In total, 127 consecutive AML patients received chemotherapy and prophylactically inhaled L-AmB during their 1st and 2nd chemotherapy cycles; 108 patients treated for AML at the same sites from 2005 to 2008 served as controls. A standardized diagnostic protocol was used and probable/proven IPA served as the primary endpoint. Diagnostic and therapeutic costs were also comprehensively analyzed and compared.

Results

A significant decrease in probable/proven IPA in the L-AmB inhalation group was observed (L-AmB 9.5%, control 23.4%, p=0.0064). Systemic antifungal therapy given at any time during the entire AML therapy decreased from 52.8% to 29.9%. Per patient equipment and drug costs for the L-AmB inhalation (1292 euro/patient) were more than compensated by a decrease in costs for diagnostics and therapeutic voriconazole use (minus 1816 euro/patient). No L-AmB inhalation related serious adverse events were observed.

Conclusion

In an unselected AML patient group, L-AmB inhalation resulted in a significant and substantial decrease in IPA and was cost saving. Now that azole resistant becomes more frequent, non-azole based prophylaxis may become an attractive strategy.

INTRODUCTION

Patients with acute myeloid leukemia (AML) treated with high-dose chemotherapy are at high risk for invasive pulmonary aspergillosis (IPA), which is associated with an increased morbidity and mortality [1,2]. Moreover, IPA increases the medical cost substantially: the mean additional per-patients costs are 8360 euros for possible IPA and 15280 euros for probable/proven IPA [3]. Given these observations and the fact that hospital resources are finite, preventing IPA is an attractive strategy to reduce mortality and IPA-related costs.

Administration of aerosolized liposomal amphotericin-B (L-AmB) is a promising candidate in preventing IPA. *Aspergillus fumigatus* conidia are inhaled and germinate in the lungs; therefore delivering aerosolized L-AmB directly to this site of infection may prevent IPA. A significant decrease in IPA was shown when inhaling L-AmB compared to placebo in a randomized controlled trial (RCT) [4]. No impaired lung function measurements or systemic side effects due to L-AmB inhalations were observed [5].

In 2008, prophylactic L-AmB inhalation was implemented as standard of care for AML patients of the Erasmus University Medical Center (Rotterdam, the Netherlands). In this prospective cohort study, the efficacy and cost-effectiveness of L-AmB inhalations on the incidence of IPA were evaluated in an unselected real-life AML population.

METHODS

Patient population

This study was performed at the Erasmus University Medical Center, a university referral hospital in Rotterdam, the Netherlands. We studied hospitalized patients with newly diagnosed or relapsed AML, myelodysplastic syndrome (MDS) with excess of blasts or chronic myeloid leukemia (CML) in blast crisis, who were aged ≥18 years and were treated during the period of September 2008 through August 2012. Patients were included after providing written informed consent. All patients were treated with remission induction and consolidation chemotherapy according to the Hemato-Oncology voor Volwassenen Nederland (HOVON) guidelines [6,7]. The following information was obtained: age, sex, number of chemotherapy cycles, type of hematopoietic stem cell transplantation (HSCT), duration of neutropenia, mortality and invasive fungal disease (IFD). Patients were excluded if they had proven, probable or possible IPA before start of the prophylactic inhalation therapy. The control group consisted of 108 historical patients with AML, MDS or CML treated with high-dose chemotherapy during the period of April 2005 through April 2008. Control patients did not receive prophylactic inhalation therapy and were not included in the L-AmB inhalation group if hematologic disease

relapsed on a later point in time. Both groups received prophylaxis with oral fluconazole 400 milligrams daily (or intravenous when oral intake was impossible). The institutional review board approved the study. According to the diagnostic protocol for evaluation of neutropenic fever, patients underwent high-resolution computed tomography (HRCT) at day 5 of unexplained fever despite treatment with antibiotics. HRCT was repeated 5-7 days later if fever persisted. Patients with intrapulmonary lesions underwent bronchoscopically-guided bronchoalveolar lavage (BAL) of the most representative lung lesion. BAL fluid was cultured for bacteria, mycobacteria and fungi, as well as measurement of galactomannan antigen levels. A galactomannan level of ≥0.5 in BAL fluid was considered positive. If bronchoscopy was impossible to perform because of the location or small size of the lung lesion and serum galactomannan was negative (<0.5), a biopsy of the lung lesions was performed if feasible. Voriconazole was the first-line therapy for IPA.

L-AmB inhalation

Prophylactic inhalation of 12.5 milligrams L-AmB (AmBisome; Gilead Sciences Europe Ltd., Uxbridge, UK) was used twice a week and was initiated at the start of the 1st remission induction chemotherapy cycle. Nebulization of L-AmB was performed with an adaptive aerosol delivery (AAD) system (Prodose® AAD nebuliser from 2008 to 2011, Akita® AAD nebuliser from 2011; Romedic, Meerssen, the Netherlands). Both are advanced nebulizer systems that adapt to individual breathing patterns, nebulizing only during inspiration and therefore the intrapulmonary delivery of the prescribed dose was guaranteed. They generate particles with a mean diameter of 1.9 µm; optimal deposition in the peripheral lung regions is therefore ensured. Inhalation was continued until neutrophil recovery, which was defined as two consecutive neutrophil counts ≥0.2 x10^9/l or one ≥0.5 x10^9/l, after which patients were discharged from the hospital. It was re-initiated during the next chemotherapy cycle until neutrophil recovery. Inhalation therapy was not continued during autologous or allogeneic HSCT following chemotherapy.

IPA classification

IPA was categorized according to the updated criteria of the European Organization for Research and Treatment of Cancer Mycosis Study Group (EORTC/MSG) [8]. Neutropenic patients are considered to have possible IPA if a new and otherwise unexplained well-defined intrapulmonary nodule (with or without halo sign), an air-crescent sign, or a cavity within an area of consolidations is radiologically documented. Probable IPA is diagnosed when on top of these radiological findings microbiological proof of *Aspergillus fumigatus* infection is documented by galactomannan antigen detection or cultures of *Aspergillus fumigatus*. Proven IPA is defined as histopathologic evidence of invasive mold infection and microbiological proof of *Aspergillus fumigatus* infection. Patients with more than 1 episode of IPA during hematological treatment were classified accord-

ing to the highest diagnostic IPA category (e.g., a patient with possible IPA during the 1st cycle of chemotherapy but probable IPA at a later point in time was categorized as having probable IPA). In addition, patients who could not be categorized into possible, probable or proven IPA but were treated with antifungal therapy were subdivided into the following categories: patients with nonspecific intrapulmonary abnormalities with positive galactomannan antigen detection, patients with nonspecific intrapulmonary abnormalities with negative galactomannan antigen detection, and patients with normal CT and negative galactomannan antigen detection. IFD was defined as a culture or biopsy proven infection with a yeast or non-*Aspergillus* mold.

Primary endpoint

The primary endpoint was the incidence of proven or probable IPA during 1st and 2nd cycles of chemotherapy until 28 days after neutrophil recovery.

Secondary endpoints

Secondary endpoints were (1) the incidence of proven or probable IPA 12 weeks after the end of all hematological treatment (including HSCT) (2) the incidence of proven, probable or possible IPA 12 weeks after at the end of all hematological treatment (including HSCT) (3) the incidence of proven, probable or possible IPA during the 1st and 2nd cycles of chemotherapy (4) overall and IPA-related (=attributable) mortality 12 weeks after recovery from neutropenia from the last chemotherapy cycle.

Cost calculations

All costs were studied from a hospital perspective. Costs taken into account were the total hospital-based costs per patient. We distinguished diagnostic costs, costs for medical treatment, costs for hospital stay, and costs for the transfusion of blood products. Diagnostic costs taken into account were radiological imaging, microbiological investigations (blood cultures, staining and cultures of BAL fluid specimens, and galactomannan antigen measurement in serum or BAL fluid specimen) and other diagnostics, e.g. bronchoscopy, pathology, colonoscopy, gastroscopy, echocardiogram. Costs of the medical treatment included costs for antifungal and antibiotic treatment, and costs for L-AmB inhalations. Costs of transfusions included costs for erythrocytes, thrombocytes, and/or fresh frozen plasma. Included extramural costs were all costs mentioned above when they occurred *between* the 1st and 2nd cycle of chemotherapy. The extramural costs *after* the 2nd chemotherapy cycle were limited to antifungal costs only and no longer than 4 weeks after discharge of the 2nd chemotherapy cycle. This was done so to avoid bias as patients that subsequently undergo a 3rd chemotherapy cycle will get this 3rd cycle (and its accompanying diagnostics and transfusion costs) within 1 or 2 weeks after the 2nd chemotherapy cycle, while an allogeneic transplantation will generally take place

later in time. The evaluation was patient based and started from day 1 of hospitalization of the 1st cycle of high dose chemotherapy for newly diagnosed or relapsed AML, MDS with excess of blasts or CML in blast crisis, which means that for each patient the costs of the 2 consecutive treatment episodes were totaled.

In the Netherlands, a detailed fee-for-service system is used for the remuneration of the medical interventions and diagnostic procedures, enabling the calculation of the costs. Therefore, medical costs were calculated by multiplying the volumes of health care use per patient with the corresponding official Euro unit prices for each diagnostic or therapeutic procedure. For drugs, the actual number of milligram administered was multiplied by the costs per milligram, as charged by the hospital pharmacy. The costs for inpatient days were calculated by multiplying the number of days with the unit price as charged for a nursing ward or intensive care unit (ICU), counting the hotel costs only.

To be able to compare the costs between groups in an unbiased way, the cost calculations for the primary analysis were limited to the first two chemotherapy cycles. The reasons that we could not include the AML therapy that followed (e.g. 3rd chemotherapy cycle, HSCT) are threefold. Firstly, over the 7 years of the study period the risk classification of AML changed substantially which resulted in a shift of patients from the intermediate to the high risk AML category. The consequence is that more patients in the L-AmB group underwent an allogeneic HSCT (36% versus 26%). Secondly, if L-AmB inhalation is indeed effective and therefore reduces the incidences of IPA, it will also have an impact on the AML therapy given after the 1st and/or 2nd chemotherapy cycle because patients that have an IPA during the 1st or 2nd chemotherapy cycle will be less likely to undergo an allogeneic HSCT. Thirdly, prophylaxis with L-AmB inhalations was only given to patients admitted to the hospital during AML therapy. During the 1st and 2nd chemotherapy cycles all patients stayed in the hospital until neutrophil recovery. In contrast, only part of the patients stayed in the hospital for the 3rd chemotherapy cycle.

Statistical analysis

Baseline characteristics and incidence of IPA and IFD were tested with the independent sample t-test or Fisher's exact test as appropriate. Cost difference between the control group and L-AmB group were analysed using the Mann-Whitney U test. Since cost data per patient (but not per day care) are typically highly skewed, we used nonparametric bootstrap techniques to derive a 95% confidence interval for the differences in distributions of the direct medical costs. For the analysis of IPA incidence during all AML therapy (including 3rd chemotherapy cycle or HSCT), patients were censored 3 months after hospital discharge from the last AML treatment episode. For the primary endpoint a 2-sided P value <0.05 was considered to be statistically significant. Analyses were done with SPSS version 20. GMC, SP and BJAR analyzed the data.

RESULTS

From September 2008 through August 2012, 127 patients in the L-AmB group received high dose chemotherapy or a HSCT during 310 treatment episodes. 226 of these were for the 1st or 2nd cycle of chemotherapy. The control group consisted of 108 historical patients who were treated during the period of April 2005 through April 2008 and had in total 262 treatment episodes. 200 of these were for the 1st or 2nd cycle of chemotherapy. Two patients, 1 of the control group and 1 of the L-AmB group, could not be included in the cost-calculation population as they had an unusual combination of AML therapy during 1 hospital admission (1 patient had the 1st, 3rd chemotherapy cycle and HSCT in 1 treatment episode, and 1 patient had the 1st chemotherapy cycle and HSCT in 1 treatment episode). From 3 patients only the 1st chemotherapy cycle could be included in the cost-calculation population as they had their 2nd chemotherapy cycle combined with an allogeneic HSCT in the same hospital admission (1 L-AmB and 2 controls). Baseline characteristics of the study groups are summarized in table 1.

Table 1. Characteristics of the cohort of 233 patients with acute myeloid leukaemia-myelodysplastic syndrome treated with high dose chemotherapy.

Characteristics	Control group n = 108	L-AmB inhalation group n = 127	p-value
Age, mean years (range)	52.1 (20.2 - 76.9)	55.7 (20.4 - 79.7)	0.051
Male gender, n (%)	61 (57.0%)	67 (53.2%)	0.5983
Diagnosis, n (%)			>0.1
Acute myeloid leukaemia	90 (83.3%)	113 (89.0%)	
Myelodysplastic syndrome with excess of blasts	18 (16.7%)	12 (9.5%)	
Chronic myeloid leukaemia in blast crisis	0	2 (1.6%)	
Mean duration neutropenia per chemotherapy treatment[a], n (%)			0.1
<10 days	0	1 (0.8%)	
≥10 – 20 days	14 (13.2%)	17 (13.6%)	
≥20 days	92 (86.8%)	107 (85.6%)	

[a] Neutrophil count <0.5 x10^9/l or leucocyte count <1.0 x10^9/l.

The incidence of IPA during the 1st and 2nd cycles of chemotherapy is given in table 2. Twenty-five patients (23.4%) in the control group developed probable or proven IPA versus 12 patients (9.5%) in the L-AmB group, a significant decrease in probable or proven IPA (p=0.0064). In a separate analysis, the incidence of probable or proven IPA according to treatment episode was analysed for the entire cohort of 235 patients and included all IPA cases diagnosed until 3 months after all therapy had been completed, including HSCT if applicable (table 3). During the 1st chemotherapy cycle, 16 of 108 patients in the control group developed probable or proven IPA versus 10 of 127 patients in the L-AmB group (p=0.1). In

total, 92 patients in the control group received the 2nd chemotherapy cycle and 99 patients in the L-AmB group. During the 2nd chemotherapy cycle, control patients developed IPA significantly more often than L-AmB patients (p=0.0246). The difference in IPA numbers in table 2 versus table 3 is explained by the fact that 6 patients with IPA were excluded from table 2 (2 patients with unusual combination of chemotherapy in 1 hospital stay as mentioned above, 1 patient receiving the 2nd chemotherapy cycle combined with HSCT in 1 hospital stay, and 3 patients who developed IPA during or shortly after their HSCT).

Table 2. Incidence of IPA and IFI during 1st and 2nd cycles of chemotherapy according to control versus L-AmB inhalation group.

Category	Control group n = 107		L-AmB inhalation group n = 126		p-value
	Count	%	Count	%	
IPA					
Probable or proven IPA	25	23.4%	12	9.5%	0.0064
No IPA and no antifungal therapy	54	50.5%	92	73.0%	0.0004
Possible/probable/proven	34	31.8%	18	14.3%	0.0014
Possible IPA (specific abnormalities on CT but culture plus antigen negative)	9	8.4%	6	4.8%	
Treatment for nonspecific abnormalities on CT, culture or antigen positive	0		3	2.4%	
Treatment for nonspecific abnormalities on CT, culture or antigen negative	10	9.3%	10	7.9%	
Empirical therapy (CT normal, antigen negative)	9	8.4%	3	2.4%	
IFD					
No IFD	101	94.4%	120	95.2%	1.0000
Candida spp.	2	1.9%	3	2.4%	
Mucor spp.	2	1.9%	3	2.4%	
Other IFD[a]	2	1.9%	0		

NOTE. IPA, invasive pulmonary aspergillosis. IFD, invasive fungal disease, excluding *Aspergillus spp*. CT, computed tomography.

[a] Other IFD, including *Cladosporium-speciës*, undefined fungal species.

Table 3. Incidence of proven/probable invasive pulmonary aspergillosis (IPA) according to treatment.

Treatment	Control group n = 108	L-AmB inhalation group n = 127	p-value
Overall	28/108	15/127	0.0066
1st chemotherapy[a]	16/108	10/127	0.0994
2nd chemotherapy[a]	11/92	3/99	0.0246
3rd chemotherapy	0/34	0/38	NA
Allogeneic HSCT	1/28	2/46	1.0000

NOTE. HSCT, hematopoietic stem cell transplantation. All 235 patients were used for this analysis, included patients who underwent HSCT.

[a] Patients who had their 1st or 2nd chemotherapy cycle combined with another chemotherapy cycle or HSCT and developed proven/probable IPA are counted in the initial chemotherapy group.

No difference in the incidence of IFD other than *Aspergillus* species was seen (table 2). During the 1st and 2nd chemotherapy cycle, systemic antifungal therapy decreased from 49.5% to 27.0% (table 2). Systemic antifungal therapy given at any time during the entire AML therapy decreased from 52.8% to 29.9%. We did not observe any major safety issues and no treatment-related serious adverse events were seen. The incidence of ICU admission during the 1st or 2nd chemotherapy cycle was comparable in both groups (20 in controls, 23 in L-AmB). None of the ICU admissions in the L-AmB group could be related to L-AmB inhalation.

A detailed overview of all medical costs is given in table 4. The control group had higher costs related to diagnostics (1101 euro/patient, p<0.0001) and related to voriconazole therapy (715 euro/patient, p=0.0031) in comparison to the L-AmB group. Therefore, the costs per patient for nebulization equipment and L-AmB drug acquisition (1292

Table 4. Mean medical costs (€) per hospital admission and per patient, control versus L-AmB inhalation group.

Cost category	Hospital admission for chemotherapy cycle								p-values
	Control group				L-AmB inhalation group				
	1 n = 94	2 n = 77	1+2[d] n=13	Total mean cost n=107	1 n = 111	2 n = 83	1+2[d] n=15	Total mean cost n = 126	Total mean costs
Diagnostics									
Total	1625	1561	3789	3011	1237	963	1557	1910	0.0000
Radiologic	622	539	1467	1113	502	345	588	739	0.0000
Microbiologic	826	861	1801	1564	580	543	817	966	0.0000
Other[a]	177	161	521	334	155	76	152	205	0.0082
Medication									
Total	2629	4251	7558	6287	4371	6107	4267	7530	0.4325
Voriconazole[b]	1144	1429	4492	2579	815	1469	1497	1864	0.0031
L-AmB inhalation	NA	NA	NA	NA	1292	NA	1292	1292	NA
Other antifungals[c]	951	2370	1874	2769	1709	2955	647	3529	0.5524
Antibiotics	533	453	1192	939	555	389	830	844	0.1553
Hospital stay	26231	28995	45146	49395	26698	26227	41994	45795	0.1046
Transfusions	7276	7071	15873	13409	7716	6383	11645	12388	0.1113
Total costs	37761	41878	72366	72102	40022	38388	59463	67624	0.2806

NOTE. NA, not applicable. Because costs are typically highly skewed, p-values were derived from 1000 non-parametric bootstrap samples drawn with replacement.

[a] Other diagnostic costs (e.g., include bronchoscopy, pathology, colonoscopy, gastroscopy and echocardiogram).

[b] Voriconazole first 8 days intravenous, followed by oral voriconazole if oral intake was possible at that time.

[c] Increased other antifungal costs in L-AmB group were caused by intravenous L-AmB use in 6 patients (median 38 days).

[d] Group of patients who had their 1st and 2nd chemotherapy cycle in one hospital admission.

euro/patient) were more than compensated by the significant decrease in per patient costs of diagnostics and voriconazole therapy. The overall treatment costs per patient were 4478 euro/patient less in the L-AmB group. This was not significantly different from the control group because extreme outliers mainly drove this difference.

IPA-related death, defined as death within 6 weeks after IPA diagnosis was seen in 2 patients (1.9%) in the control group versus 3 (2.4%) in the L-AmB group. During the 1st or 2nd chemotherapy cycle, 14/107 patients (13.1%) died in the control group versus 15/126 (11.9%) in the L-AMB group. The overall mortality 3 months after the end of all therapy was 19 (17.6%) in the control group versus 23 (18.1%) in the L-AmB group.

DISCUSSION

In 2008, the efficacy of inhaled L-AmB for the prevention of IPA was demonstrated in the context of a RCT. In 2010, this resulted in a BI recommendation for the use of this intervention in the European Conference on Infections in Leukaemia (ECIL) guidelines for AML patients during remission induction and consolidation chemotherapy [9]. It is difficult to predict how interventions work in real-life outside the context of a RCT. In this study we established the external validity of the RCT outcomes in a real-life AML patient population. Overall, the incidence of IPA was significantly higher in historical control patients than patients inhaling aerosolized L-AmB (23.4% versus 9.5%). No specific exclusion criteria were used apart from an already established IPA diagnosis at the time of AML diagnosis for which antifungal therapy instead of prophylaxis needed to be initiated. Apart from its efficacy, the use of L-AmB inhalation seems to be cost saving in regard to diagnostics and voriconazole therapy. These cost-savings more than compensated for the costs related to L-AmB inhalation itself.

The difference in IPA incidence was more pronounced during the 2nd than the 1st chemotherapy cycle. This may be the result of undiagnosed IPA at baseline in a small number of patients for which systemic therapy rather than inhalation prophylaxis should have been given. Only a study in which a high-resolution CT is performed in all patients at baseline to exclude patients with pre-existent intrapulmonary lesions could confirm this. One wonders if prophylaxis with a broad spectrum azole during the first 7 to 10 days may further reduce the IPA incidence during the 1st chemotherapy cycle.

We did not observe any major safety issues. The incidence of ICU admissions was comparable in both groups and none of the ICU admissions in the L-AmB group could be related to L-AmB inhalation. Still, an easier and more convenient way to get amphotericin-B inside the lungs would be very welcome. An inhalation amphotericin-B powder was developed several years ago, has favourable pharmacokinetic properties

and seems effective in an animal model [10]. Unfortunately, as far as we know no clinical trials are planned.

This study has some limitations. Considering that medicine evolves quickly, a time related bias might occur when controls are not from the same calendar year as cases. However, no considerable change has occurred in the chemotherapeutic agents that were used in the 1st and 2nd chemotherapy cycles during the study period of 2005 till 2012. Secondly, the diagnostic procedures have remained unchanged throughout the study and therefore diagnostic bias is unlikely.

We observed no difference in overall mortality 3 months after all therapy (including HSCT if applicable) between the L-AmB and control group. However, this comparison is likely to be confounded by indication as the chances of getting an allogeneic HSCT is less likely for patients with IPA and higher for patients in more recent years (due to a change in the AML risk classification). We also did not observe a decrease in the mortality attributable to IPA. This is not surprising, given the fact that IPA related mortality has decreased substantially with voriconazole therapy. It was estimated to be as low as 10% in a comparable AML patient population [3]. It is therefore not surprising that, despite the relatively large study population, no significant reduction in overall mortality was demonstrated.

Other prophylactic regimes to prevent IA have been studied in hematological patients with prolonged chemotherapy-induced neutropenia and in allogeneic HSCT patients. Prophylaxis with posaconazole showed a significant decrease in IFD (including IA) in both of these patient groups [11,12]. Voriconazole and itraconazole as prophylaxis have been investigated in allogeneic HSCT patients. Voriconazole showed no difference in the incidence of IFD in comparison to fluconazole in patients undergoing allogeneic HSCT [13]. Itraconazole reduced the incidence of IFD [14] and invasive mold infections [15] in comparison to fluconazole. However, the itraconazole groups in these two studies suffered significantly more gastrointestinal intolerance and toxicity, limiting its success as prophylaxis. Low dose L-AmB at 50 milligrams intravenously every other day reduced the incidence of IFD (including IA) in hematological patients with prolonged chemotherapy-induced neutropenia [16]. However, the control group in this study did not receive fluconazole as anti-yeast prophylaxis. No nephrotoxicity was observed and few patients discontinued L-AmB prophylaxis because of skin rash and infusion-related fever. However, patients with renal insufficiency or liver dysfunction were excluded from participating in this study, whereas in our current study all patients were included except for those who had preexistent IA. Lastly, micafungin as prophylaxis has been studied in allogeneic HSCT patients during their neutropenic period [17]. The study showed that micafungin reduced the IFD incidence, but the reduction in IA was not statistically significant, possibly because micafungin was only administered during the hospital stay

(18 days on average). The cost-effectivity of these intravenously administered antifungals remains to be demonstrated.

Aspergillus fumigatus was cultured in five patients, all in the control group at a time when azole resistance testing was not performed as a routine procedure in our hospital. Azole susceptibility testing was performed in one of the five patients who developed probable pulmonary and cerebral aspergillosis during the 2nd chemotherapy cycle. He was treated with voriconazole and caspofungin. Despite treatment, patient deteriorated clinically and died of progressive IA. The isolate that was cultured from sputum showed resistance to itraconazole (minimum inhibitory concentration (MIC) 16 µg/ml) and voriconazole (MIC 8 µg/ml). In the new millennium, azole resistant *Aspergillus fumigatus* has become a significant problem in the Netherlands [18,19]. More recently, resistance was also observed in several other European countries [20-23]. This is probably the consequence of extensive agricultural azole use [24]. The use of L-AmB inhalation may therefore become an attractive option for the prevention of IPA when prophylactic as well as therapeutic azole use becomes less effective.

With the current study results available and together with the clinical trial data from 2008, we think it is time to move forward and study the use of L-AmB inhalation in other patients at high risk for IPA, in particular in patients with serious graft versus host disease.

Prophylactic L-AmB inhalation administered twice a week to AML or MDS patients undergoing remission induction and consolidation chemotherapy resulted in a substantial decrease in IPA incidence. Furthermore, the costs of inhalation therapy were more than compensated by a decrease diagnostic costs and voriconazole therapy costs, both of which decreased significantly.

FUNDING

This study was supported by a grant from Gilead Sciences to B.J.A. Rijnders.

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Chapter 4

Diagnostic performance of galactomannan antigen testing in cerebrospinal fluid

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J Clin Microbiol 2016;54:428-431

ABSTRACT

Introduction

Testing cerebrospinal fluid (CSF) for the presence of galactomannan (GM) antigen may help in diagnosing cerebral aspergillosis (CA). However, the use of CSF GM as a diagnostic test never been validated. We evaluated its diagnostic performance by comparing the CSF GM levels at different cut-offs in patients with probable and proven CA to those without CA.

Methods

Patients from 2 tertiary referral hospitals with suspected CA between 2004-2014 and in whom CSF GM had been determined, were selected. EORTC/MSG definitions of invasive aspergillosis and CA were used but excluding the to-be-validated-test (=CSF GM) as a microbiological EORTC/MSG criterion.

Results

The study population consisted of 44 patients (4 proven CA, 13 probable CA and 27 no CA). Of the 17 patients with CA, 15 had a CSF GM of ≥2.0. In patients without CA, 26 of the 27 had a CSF GM of <0.5 and 1 had a CSF GM of 8.2. When a GM CSF cut-off level of 1.0 was used, the sensitivity, specificity, positive and negative predictive values were 88.2%, 96.3%, 93.8% and 92.9%, respectively. The same results were found when using a CSF GM cut-off of 0.5 or 2.0.

Conclusion

GM in CSF has a high diagnostic performance for diagnosing CA and may be useful to diagnose or virtually rule out the infection without the need for a cerebral biopsy.

INTRODUCTION

Cerebral aspergillosis (CA) is a rare and often fatal invasive fungal disease (IFD) [1,2]. The diagnosis is challenging as radiological findings are non-specific and cerebrospinal fluid (CSF) cultures are only positive in less than one-third of the cases [3,4]. Moreover, obtaining brain tissue for histopathological examination, the diagnostic gold standard, is frequently not feasible. Galactomannan (GM) antigen detection in CSF is one of the microbiological criteria of the revised European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) criteria [5]. However, its diagnostic performance has been little studied. To date, only 3 studies totalling 25 patients have described the value of CSF GM, only 2 of which in patients with suspected CA [3,6,7]. In these 2 studies, CSF GM levels were higher in patients with than without CA, and sensitivity and specificity were 80% and 100% [6,7]. Based on these limited data, GM antigen test in CSF seems a promising test, even though no positive and negative predictive values (PPV, NPV) were determined, and no formal cut-off was established. This study evaluated the diagnostic performance of the Platelia™ GM antigen test (Bio-Rad, Marnes-la-Cocquette) in CSF of patients with suspected CA.

METHODS

This retrospective study was performed at the Erasmus University Medical Center (Erasmus MC) in the Netherlands and University Hospitals Leuven (UZ Leuven) in Belgium. Patients in whom a CSF GM had been performed, were considered to be "suspected CA" and were selected. In the Erasmus MC, internal medicine, haematology and paediatric patients were selected from January 2004 to March 2015. In the UZ Leuven, internal medicine and haematology patients from May 2007 to December 2014 were retained. Data on age, sex, underlying disease, microbiology and radiology results were collected. Patients were excluded if no pulmonary or cerebral radiology was present, or no serum and BAL GM had been performed.

The diagnostic performance of the GM antigen test in CSF was evaluated by comparing the GM antigen level in CSF of patients with proven or probable CA to patients without CA. Patients with proven, probable or no CA were selected in two steps. First, invasive aspergillosis (IA) was defined or ruled out according to the revised EORTC/MSG criteria [5]. To avoid inclusion of the test that we wanted to validate (CSF GM) into the gold-standard, CSF GM was removed from the microbiology criteria. To avoid overlap between the definition of IA and CA, cerebral radiology was also excluded from the clinical EORTC/MSG criteria. This was deemed necessary because otherwise patients with an

isolated serum GM ≥ 0.5 and a focal cerebral lesion (e.g. cerebral infarction) but without any other evidence of IA elsewhere would fit the probable CA definition.

Subsequently, proven or probable CA was determined in patients with proven or probable IA. IA remains uncertain in patients with possible IA or in patients with only an isolated positive microbiological criterion but no clinical or radiological criterion. Therefore, these patients were excluded from the analysis. Probable CA was diagnosed when cerebral radiological signs compatible with IFD (e.g. focal lesions, meningeal enhancement) were present on top of a proven or probable IA elsewhere in the body. Proven CA was diagnosed when cerebral pathological evidence of IA or a positive CSF *Aspergillus* culture was present on top of the IA criteria. Patients with proven or probable IA who had non-specific radiological cerebral signs (no focal lesion, no meningeal enhancement) were excluded from analysis. Patients classified as being without CA had no IA and did not have cerebral abnormalities or had a convincing alternative diagnosis for the cerebral abnormalities. Patients were only included once and those who had more than one episode of suspected CA, were classified according to the highest CA category. Per patient, only the CSF GM at diagnosis was included.

The diagnostic performance of GM antigen in CSF were evaluated by comparing the GM antigen level at different cut-offs in proven and probable CA cases to those without CA. CSF GM levels were correlated with serum levels and CSF cultures. The independent t-test or Mann-Whitney-U test was used as appropriate to compare the CSF and serum GM values (IBM SPSS Statistics, version 21).

In addition, an extra sensitivity analysis was performed to look at the diagnostic performance of CSF GM when cerebral radiology was not excluded from the clinical EORTC/MSG criteria. For this sensitivity analysis, CSF GM cut-off of 1.0 was used.

RESULTS

GM was determined in 205 CSFs of 157 patients. Eighty patients were excluded because of insufficient microbiology (n = 10), radiology (n = 47) or both (n = 23). Further, 12 patients with possible IA, 9 with an isolated microbiology criterion and 12 with cerebral findings not compatible with IFD were excluded (figure 1). Therefore, the evaluable study population consisted of 44 patients (4 proven CA, 13 probable CA and 27 no CA). Table 1 shows the clinical, radiological and microbiological findings for those with CA. Fifteen of the 17 patients with CA had CSF GM ≥ 2.0. In the patients without CA, 26 had CSF GM <0.5 and 1 had CSF GM of 8.2. When a GM CSF cut-off level of 1.0 was used, the

sensitivity, specificity, PPV and NPV were 88.2%, 96.3%, 93.8% and 92.9% (table 2). The same results were found when a cut-off of 0.5 or 2.0 was used. With increasing cut-off values of 3.0 / 4.0 / 5.0, the sensitivity decreased to 76.5 / 70.6 / 58.8% (table 3). As a sensitivity analysis, we looked at the impact of including cerebral radiology in the clinical EORTC/MSG criteria using a GM cut-off of 1.0; this decreased the sensitivity from 88.2 to 76.0% (table 4).

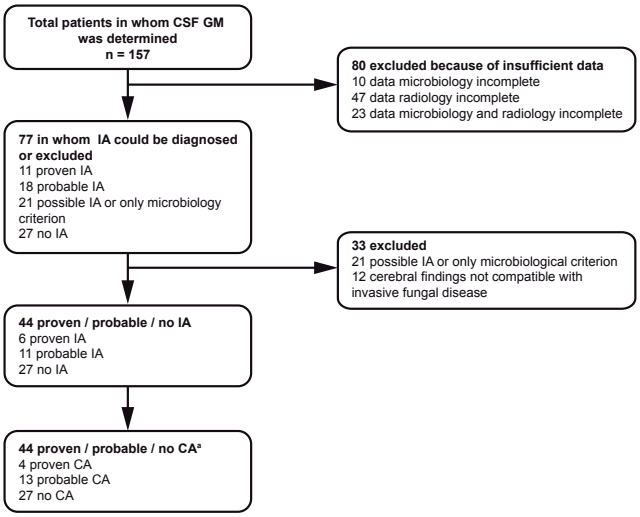

Figure 1. Flowchart of the 44 patients with suspected cerebral aspergillosis (CA).
Two patients with proven IA had histopathological evidence of IA outside the cerebrum and therefore were classified as probable CA cases. CSF, cerebrospinal fluid; GM, galactomannan; IA, invasive aspergillosis.

Of the 17 CA patients, 3 were culture positive (2 CSF and 1 biopsy) and all grew *A. fumigatus*.

Serum GM was available in 16/17 patients with CA. CSF GM was higher than serum GM in 10 patients and lower in 1 patient with probable CA (table 1). The mean GM in CSF was higher than in serum (4.89 versus 3.72; p-value = 0.27). In the patients without CA, serum GM was available in 25/27 patients. CSF GM was higher than serum GM in 4 patients, lower in 4 patients, and equal in the remaining 17 patients. The median GM was 0.1 in CSF and 0.1 in serum (p-value = 0.77).

Table 1. Clinical characteristics, radiological and microbiological findings for the patients with probable or proven cerebral aspergillosis (CA).

Patient no.	Age (y)/ sex[a]	IA[b]	CA	Underlying disease / host factor(s)[c]	Radiological findings[d]		Galactomannan level[e]			Culture[f]		Pathology
					Cerebral	Lungs	CSF	BAL	Serum	CSF / cerebral biopsy	BAL	
1	36/M	Probable	Probable	ALL, allogeneic HSCT	Multiple lesions, sinusitis	Specific	5.5	NA	5.5	Neg	NA	NA
2	67/F	Probable	Probable	AML	Multiple lesions	Specific	6.4	6.3	6.4	Neg	*A. fumigatus*	NA
3	58/M	Proven	Proven	No host factors	Multiple abscesses	Non-specific	2.3	0.1	0.2	*A. fumigatus*	Neg	Pos, cerebral biopsy
4	5/M	Probable	Probable	ALL	Multiple lesions	Specific	7.2	NA	7.2	Neg	NA	NA
5	9/F	Probable	Probable	ALL	Multiple lesions	Specific	9.9	NA	9.9	Neg	NA	NA
6	53/M	Probable	Probable	NHL	Multiple lesions	Specific	5.0	4.2	4.5	Neg	*A. fumigatus*	NA
7	63/M	Probable	Probable	Cushing's syndrome	Nodular lesion	Specific	3.8	5.6	0.2	Neg	Neg	NA
8	2/F	Proven	Proven	ALL	Multiple lesions	Normal	7.1	NA	2.9	*A. fumigatus*	NA	NA
9	69/M	Proven	Proven	Peripheral arterial disease	Meningitis with infarct	Normal	4.3	NA	NA	*A. fumigatus*	NA	Neg, cerebral autopsy
10	54/M	Probable	Probable	AML, allogeneic HSCT	Multiple lesions	Specific	5.0	0.5	0.1	Neg	*A. fumigatus*	NA
11	59/M	Probable	Probable	AML, allogeneic HSCT	Multiple abscesses	Specific	6.2	NA	6.2	Neg	NA	NA
12	30/M	Probable	Probable	AML, allogeneic HSCT	Multiple lesions	Specific	2.1	NA	2.0	Neg	NA	NA
13	68/F	Proven	Proven	Kidney transplantation	Infarct	NA	7.9	NA	7.4	Neg	NA	Pos, cerebral autopsy
14	66/M	Proven	Probable	NHL	Multiple lesions	NA	5.0	5.3	4.8	Neg	*A. fumigatus*	Pos, lung autopsy
15	67/M	Probable	Probable	AML	Semi-recent ischemia	Specific	0.1	NA	1.5	Neg	Neg	Neg, CSF cytology

Table 1. Clinical characteristics, radiological and microbiological findings for the patients with probable or proven cerebral aspergillosis (CA) (*continued*)

Patient no.	Age (y)/ sex[a]	IA[b]	CA	Underlying disease / host factor(s)[c]	Radiological findings[d]		Galactomannan level[e]			Culture[f]		Pathology
					Cerebral	Lungs	CSF	BAL	Serum	CSF / cerebral biopsy	BAL	
16	66/F	Proven	Probable	AML, allogeneic HSCT	Hypodense lesion	Non-specific	0.2	5.1	0.1	NA	*A. fumigatus*	Pos, lung biopsy
17	66/F	Probable	Probable	ALL, allogeneic HSCT	Multiple lesions	Specific	4.5	0.8	0.6	Neg	Neg	NA

[a] M, male. F, female. [b] IA, invasive aspergillosis. [c] ALL, acute lymphatic leukaemia. HSCT, hematopoietic stem cell transplantation. AML, acute myeloid leukaemia. NHL, non-Hodgkin lymphoma. [d] NA, not available. [e] CSF, cerebrospinal fluid. [f] Neg, negative. Pos, positive.

Table 2. Cerebrospinal fluid (CSF) galactomannan (GM) related to patients with suspected cerebral aspergillosis (CA).

	Patients with CA	Patients without CA	Total
CSF with positive GM (≥ 0.5 / 1.0 / 2.0)	15	1	16
CSF with negative GM (<0.5 / 1.0 / 2.0)	2	26	28
Total	17	27	44

Sensitivity	= 15 / 17 = 0.8824
Specificity	= 26 / 27 = 0.9630
Positive predictive value	= 15 / 16 = 0.9375
Negative predictive value	= 26 / 28 = 0.9286

Table 3. Diagnostic performance of galactomannan (GM) in cerebrospinal fluid (CSF) according to different cut-offs.

	Cut-off value of GM in CSF					
	0.5	**1.0**	**2.0**	**3.0**	**4.0**	**5.0**
Sensitivity (%)	88.2	88.2	88.2	76.5	70.6	58.8
Specificity (%)	96.3	96.3	96.3	96.3	96.3	96.3
PPV (%)	93.8	93.8	93.8	92.9	92.3	90.9
NPV (%)	92.9	92.9	92.9	86.7	83.9	78.8

Table 4. Sensitivity analysis of galactomannan (GM) in cerebrospinal fluid (CSF).

	Cut-off value of GM in CSF	
	GM level < 1.0	**GM level ≥ 1.0**
Proven CA	0	4
Probable CA	7	15
Possible CA	1	2
No CA	44	4
Total	52	25

For the sensitivity analysis, cerebral radiology was included in the clinical criteria of the revised European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group. Sufficient data of 77 patients were available to diagnose or exclude cerebral aspergillosis (CA). Patients with probable or proven cerebral aspergillosis (CA) were compared to patients without CA. Patients with possible CA were excluded. When a GM level of 1.0 in CSF was used, the sensitivity was 76.0% (19/25).

DISCUSSION

In this study, the GM antigen test on CSF showed a good performance for diagnosing CA in patients with proven/probable CA when using a cut-off of 0.5 to 2.0. To validate the CSF GM antigen test, we logically had to exclude CSF GM from the EORTC/MSG criterion.

This made it possible to measure the sensitivity of CSF GM to diagnose CA. As such, we found that in patients with a proven/probable CA based on culture, tissue biopsy or GM in serum or BAL in combination with a suspected radiological cerebral finding, 88.2% of the patients had a positive CSF GM, while cultures were only positive in 17.6% of the cases. As explained in the methods, we chose to remove the cerebral radiology from the clinical EORTC/MSG criteria. In a sensitivity analysis, we retained the cerebral radiology in the criteria and observed a decrease in sensitivity to 76% (CSF GM cut-off of 1.0). We think that this decrease is rather the result of more misclassifications of patients in the probable CA group. However, we cannot formally prove this.

The CSF GM antigen test is included in the revised EORTC/MSG [5]. However, only 2 studies reported on GM antigen testing in CSF in a total of 10 patients with CA. Viscoli et al. measured GM on CSF from 5 patients with proven/probable CA [7]. The median GM level was 10.52 and was significantly higher in patients with CA compared to patients without CA. Kami et al. compared different non-culture based diagnostics on CSF of 5 patients with proven CA and 11 patients with leukemic, bacterial, viral or mucor meningitis [6]. The GM antigen test was positive in 4 of the 5 CSF in patients with proven CA and negative in all patients without CA. Based on the findings of Viscoli et al. and Kami et al., the CSF GM was included in the revised EORTC/MSG definitions. Finally, Antinori et al. reviewed the literature on *Aspergillus* meningitis, which is not the same as CA, and found that CSF GM was performed in 15 of the 93 cases [3]. The median CSF GM was 6.58 with a range of 2.2 to 578. The sensitivity was 86.7%. Our study, in which 17 patients with CA and 27 without CA were included, confirms that CSF GM is a useful test to rule in or rule out CA.

Among the patients without CA in our study, there was 1 patient with positive CSF GM of 8.2. This patient had a cerebral abscess on magnetic resonance imaging. As he did not have any other localisations of IA, and no positive culture or brain biopsy he was classified as having no CA according to our study criteria in which we excluded CSF GM. However, according to the revised EORTC/MSG, this patient had probable IA and was treated with voriconazole. He died 17 days later. Because no autopsy was performed, we cannot exclude that this patient had CA, but this is an intrinsic problem when a new diagnostic test is being validated.

This study has limitations. The study was performed retrospectively and for logistical reasons, patients could be selected only from the departments where 1 of the co-authors worked. Secondly, we excluded patients who according to EORTC/MSG had a possible IA or had an isolated positive microbiological criterion. Including these uncertain IA cases as suffering from IA (or the opposite) would unavoidably lead to an uncertain number of misclassification. Therefore, to make a validation of GM antigen testing in CSF possible, we could only but exclude them.

In conclusions, GM detection in CSF showed a good diagnostic performance when a cut-off of 0.5 to 2.0 was used, and using GM in CSF, CA can be diagnosed or virtually ruled out without the need for cerebral biopsy.

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Chapter 5.1

Direct detection of *Aspergillus* and azole resistance of *Aspergillus fumigatus* on bronchoalveolar lavage fluid. Validation of a new *Aspergillus* real-time PCR.

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J Clin Microbiol 2015;53:868-874

ABSTRACT

Introduction

Azole resistance in *Aspergillus fumigatus* is increasingly reported. We describe the validation of AsperGenius®, a new multiplex real-time polymerase chain reaction (PCR) assay consisting of two multiplex real-time PCRs: one which identifies the clinically relevant *Aspergillus* species, and one which detects the TR_{34}, L98H, T289A, Y121F mutations in *Cyp51A* and differentiates susceptible from resistant *A. fumigatus* strains.

Methods

The diagnostic performance was tested on 37 bronchoalveolar lavage (BAL) samples from hematology patients and on 40 BAL samples from intensive care unit (ICU) patients using BAL galactomannan ≥1.0 or positive culture as the gold standard for the presence of *Aspergillus*.

Results

In the hematology and ICU groups combined, there were 22 BAL samples with IA (2 proven, 9 probable and 11 non-classifiable). Nineteen of the 22 BAL samples were positive according to the gold standard. The optimal cycle threshold value for the presence of *Aspergillus* was <36. Sixteen of the 19 BAL samples had a positive PCR (2 *Aspergillus* species and 14 *A. fumigatus*). This resulted in a sensitivity, specificity, positive and negative predictive value of 88.9%, 89.3%, 72.7% and 96.2% for the hematology group and 80.0%, 93.3%, 80.0% and 93.3% in the ICU group, respectively. The *Cyp51A* real-time PCR confirmed 12 wildtype and 2 resistant strains (1 TR_{34}/L98H and 1 TR_{46}/Y121F/T289A mutant).

Conclusion

The AsperGenius® multiplex real-time PCR allows for a sensitive and fast detection of *Aspergillus* species directly in BAL samples. More importantly, this assay detects and differentiates wildtype from resistant strains even if BAL cultures remained negative.

INTRODUCTION

Aspergillus fumigatus is the most frequent cause of invasive mould infections in immunocompromised patients. Its mortality varies substantially and depends on patient characteristics and the extent of disease. Mortality in intensive care unit (ICU) patients with invasive aspergillosis (IA) can be as high as 90% [1]. In hematology patients, a relatively low mortality is observed when the diagnosis is made early and treatment with voriconazole, the current standard of care [2], is initiated promptly [3]. In 2002, the landmark study by Herbrecht *et al*. showed that treatment of IA with voriconazole resulted in an improved survival [4]. However, a series of recent publications described the appearance of azole resistance in *A. fumigatus* [5-10]. This resistance is caused by a mutation in the *Cyp51A* gene of *A. fumigatus* at position 98 (L98H) together with a 34-basepair tandem repeat (TR) in the promoter region (TR_{34}). *Cyp51A* encodes for cytochrome p450 sterol 14α-demethylase, the target of azoles. The majority of these mutated strains were cultured from patients never exposed to azoles. It is assumed that resistance development is caused by environmental azole exposure [11]. More recently, van der Linden *et al*. described a second mutation, a 46-basepair TR combined with the point mutations Y121F and T289A [12]. In this study, 47 of 921 patients (5.1%) were diagnosed with TR_{34}/L98H and 13 (1.4%) with the TR_{46}/Y121F/T289A mutation. Occasionally, other mutations have also been described [13-16]. Infections with azole resistant strains are associated with a very high mortality [17].

Currently, the absence of a non-culture based, fast and readily available azole susceptibility testing method compromises the identification of azole resistance. This is a major limitation as the mortality of IA increases substantially when the initiation of adequate therapy is delayed [18]. Furthermore, most *Aspergillus* infections are diagnosed indirectly using galactomannan (or beta 1-3 d-glucan) testing because cultures remain negative in most patients. Therefore, even if culture based azole resistance testing would become broadly available, this would only be helpful in a subset of patients.

This study describes the laboratory and first clinical validation of AsperGenius®, a new *Aspergillus* real-time polymerase chain reaction (PCR) that detects *Aspergillus* species directly from bronchoalveolar lavage (BAL) and simultaneously identifies the most prevalent *Cyp51A* mutations in *A. fumigatus*.

METHODS

This retrospective study was performed at the Erasmus University Medical Center in the Netherlands. The following information was obtained: age, sex, mortality, underlying disease, reason for ICU admission, hospital admission duration, presence and treatment

of IA. For the *Aspergillus* PCR, we used stored BAL samples of historical patients. BAL samples of hematological and ICU patients were selected because these patients are at high risk for IA.

Processing of BAL samples

BAL samples from ICU patients (1-2 ml) were incubated with 0.1 M dithiothreitol (DTT) to reduce viscosity. This was not needed for BAL samples from neutropenic hematology patients. Subsequently, all BAL samples were centrifuged at high speed (10 min at 13400 g). After centrifugation, the supernatant and the pellet were processed in different ways. The supernatant was added to 2 ml NucliSENS® lysis buffer (BioMérieux, Boxtel, the Netherlands) and incubated for 10 min at room temperature. An Internal Control (IC) was added to the BAL to monitor PCR inhibition, DNA-extraction efficiency and manual handling errors. The pellet was transferred to green bead tubes (Roche Diagnostics, Indianapolis, USA) and 500 µl NucliSENS® lysis buffer was added together with 5 µl of IC. The pellets were subsequently bead-beaten in a MagNA Lyser instrument (Roche Diagnostics) for 45 sec at 6500 rpm. Proteinase K (Roche Diagnostics) was added and incubated for 10 min at 65 °C, and subsequently inactivated for 10 min at 95 °C. After centrifugation, the supernatant of the lysed pellet suspension was transferred to a new tube. DNA from both supernatant and pellet was extracted with the NucliSENS® miniMAG magnetic extraction (BioMérieux) according to the manufacturer's instructions. The DNA from both the pellet and supernatant were tested separately.

AsperGenius® multiplex real-time PCR assay

The AsperGenius® multiplex real-time PCR assay (PathoNostics, Maastricht, the Netherlands) was used for the detection of *Aspergillus* species and the identification of prevalent mutations conferring resistance against triazoles. The AsperGenius® species multiplex assay allows for specific detection of *A. fumigatus* complex, *A. terreus* and *Aspergillus* species by targeting the 28S rRNA multicopy gene. The *A. fumigatus* probe detects the most relevant species of the *Fumigati* complex: *A. fumigatus, A. lentulus, A. udagawae* and *A. viridinutans.* The *Aspergillus* species probe specifically detects *A. fumigatus, A. terreus, A. flavus* and *A. niger.* In addition the *A. nidulans* could be detected based on sequence information. Detection of the IC is included. The AsperGenius® resistance multiplex assay targets the single copy *Cyp51A* gene of *A. fumigatus* and detects TR_{34}, L98H, Y121F and T289A to differentiate wildtype from mutant *A. fumigatus* strains via melting curve analysis. The real-time PCR was performed according to the manufacturer's instructions. Detection of four different fluorescent labels (emission spectra: 495 nm, 530 nm, 598 nm, 645 nm) was enabled by using the Rotor-Gene Q (Qiagen, Heidelberg, Germany) for all experiments. The real-time PCR assay was first validated on DNA of 131 *A. fumigatus* cultures including resistant strains (Erasmus University Medical Center) before testing BAL samples.

These strains were identified to the species level on morphology and by sequencing of the internal transcribed spacer region. Furthermore, the assay was tested for cross reactivity with species selected on their prevalence in the respiratory tract and/or their genomic similarity. The specificity was tested for the following species: *P. marneffei, P. chrysogenum, Fusarium* species, *Scedosporium* species, *R. oryzae*, *S. cerevisae, C. neoformans, C. albicans ,C. lustaniae, C. krusei*, *C. dubliniensis, C. guilliermondii, C. tropicalis, C. glabrata, C. parapsilosis, P. jirovecii*, *B. pertussis, E. coli, H. influenzae, M. catarrhalis, P. aeruginosa, S. aureus*, *S. pneumoniae, K. pneumoniae, C. pneumoniae, L. pneumoniae*, *M. pneumoniae.*

Each extracted BAL sample was tested in duplicate and a no template control (blank) was included in each run to exclude contamination. A sample was considered positive when one of the duplicates showed increased fluorescence above the threshold. Synthetic single-stranded DNA targets (IDT, Heverlee, Belgium) were included in the assay as a standard (positive control) for the melting peaks. These positive control sequences were tested simultaneously with the BAL samples to determine if the melting peak represents wildtype or resistant *A. fumigatus* strains.

Gold standard

BAL samples with a positive galactomannan (≥ 1.0) or positive BAL or sputum cultures for *Aspergillus* were selected as the gold standard for positivity. True negatives were BAL samples with a negative BAL galactomannan in combination with a negative culture from BAL or sputum. BAL samples with only a positive serum galactomannan (≥ 0.5) were considered to be negative as there was no microbiological evidence of the presence of *Aspergillus*.

Classification of IA

The European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) criteria for IA were used to classify patients into *possible*, *probable* or *proven* IA. A patient is considered to have ***possible IA*** if a new and otherwise unexplained well-defined intrapulmonary nodule (with or without halo sign), an air-crescent sign, or a cavity within an area of consolidations is radiologically documented in an immunocompromised host. ***Probable IA*** is diagnosed when on top of these radiological findings microbiological proof of *A. fumigatus* infection is documented by galactomannan antigen detection (Platelia™ Bio-Rad inc.) or positive cultures of *A. fumigatus.* Galactomannan was considered positive in BAL fluid if ≥ 1.0 and in serum when ≥ 0.5. ***Proven IA*** is defined as histopathologic evidence of invasive mould infection and microbiological proof of *A. fumigatus* infection. Immunocompromised patients who had a positive galactomannan test but who could not be categorized into probable IA, because the radiology of the lungs was non-specific, were referred as ***non-classifiable IA***, a category not included in the EORTC/MSG definitions. In clinical practice, these patients

are treated similarly to patients with probable IA because the outcome of these patients is comparable to patients with probable IA [19]. Note that most ICU patients with a clinical diagnosis of IA will fall into this non-classifiable category because in contrast to the neutropenic patients, the findings on high-resolution computed tomography or chest X-ray in ICU patients with IA is mostly non-specific [1].

PCR cut-off analysis

To determine the most appropriate *Aspergillus* PCR cut-off for clinically significant positivity, we first analysed the cycle threshold (Ct) values of the *Aspergillus* PCR on 37 BAL samples of 34 hematological patients. Three patients in the hematology group underwent a BAL twice at different times and therefore contributed two BAL samples each. The lowest Ct value of the pellet or supernatant was used. The optimal cut-off was assessed with receiver operator characteristic (ROC) curves. Areas under the curve (AUC) were estimated to determine discriminatory power (IBM® SPSS® statistics, version 21). In a second set of 40 BAL samples from 39 ICU patients we confirmed the usefulness of the cut-off that was obtained in the hematology population. The sensitivity, specificity, positive and negative predictive values (PPV, NPV) were calculated for the two groups separately and combined.

RESULTS

The AsperGenius® multiplex real-time PCR assay was first tested on 131 *A. fumigatus* strains. Based on the PCR assay, 17 resistant strains were identified which all carried the TR_{34}/L98H mutation. These data were confirmed by sequencing the *Cyp51A* regions. Furthermore, the specificity of the assay was tested with species selected on their prevalence in the respiratory tract and/or their genomic similarity. No cross-reactivity was observed for *the A. fumigatus*, *A. terreus* and the resistant probes. Limited cross-reactivity was observed with the *Aspergillus* species probe for *R. oryzae and P. chrysogenum* that resulted in false positive signals when using a 1000 times higher DNA load (50 picogram) than the A. fumigatus DNA load needed to get a PCR positive results with a CT of 36 (50 fentogram or 2 DNA copies/ml).

In the hematology and ICU groups combined, there were 22 BAL samples with proven, probable or non-classifiable IA (table 1). More detailed information on the complete set of hematology and ICU BAL samples can be found in the supplement. There were three patients with non-classifiable IA, one in the hematology group and two in the ICU group who had negative BAL galactomannan and culture, but had a positive serum galactomannan. Because BAL galactomannan tests and cultures were negative, there was no microbiological evidence that *Aspergillus* was present in these BAL samples. Therefore, these three BAL samples were counted as negatives in the statistical analysis.

Table 1. Clinical characteristics, radiological findings and results of the galactomannan and AsperGenius® multiplex real-time polymerase chain reaction (PCR) for 22 bronchoalveolar lavage (BAL) samples of patients with proven, probable and non-classifiable invasive aspergillosis (IA).

Patient no.	Clinical setting[a]	IA diagnosis[b]	Underlying disease/ host factor(s)[c]	Radiological findings	BAL or sputum culture[d]	Pathology[e]	AsperGenius® multiplex real-time PCR[f]						
							Galactomannan level		Ct value of *Aspergillus* species PCR		Ct value of *A. fumigatus* PCR		*Cyp51A* PCR
							Serum	BAL	Supernatant	Pellet	Supernatant	Pellet	
1	HEM	Probable	MM, allogeneic HSCT	Specific	Pos	NA	0.2	1.5	Pos (29)	Pos (28)	Pos (29)	Pos (29)	WT
2	HEM	Probable	AML	Specific	Neg	NA	NA	1.3	Pos (32)	Pos (34)	Pos (33)	Pos (35)	WT
3	HEM	Probable	AML	Specific	Pos	NA	0.3	0.2	Pos (33)	Pos (27)	Pos (33)	Pos (28)	WT
4	HEM	Probable	AML	Specific	Neg	NA	1.0	7.1	Pos (29)	Pos (31)	Pos (30)	Pos (32)	WT
5	HEM	Non-classifiable	APL	Not specific	Neg	NA	0.1	1.1	Neg	Neg (39)	Neg	Neg	
6	HEM	Probable	AML	Specific	Neg	Neg	0.4	2.0	Pos (31)	Pos (32)	Pos (33)	Pos (33)	TR_{34}/L98H
7	HEM	Non-classifiable	CLL, allogeneic HSCT	Not specific	Pos	NA	1.3	5.1	Pos (27)	Pos (27)	Pos (29)	Pos (29)	WT
8	HEM	Probable	MM, allogeneic HSCT	Specific	Neg	NA	0.9	5.7	Pos (33)	Pos (35)	Neg (39)	Neg (42)	
9	HEM	Non-classifiable	AML	Not specific	Neg	NA	0.6	0.1	Neg	Pos (34)	Neg	Neg	
10	HEM	Non-classifiable	CLL	Not specific	Pos	NA	0.1	1.3	Pos (35)	Pos (32)	Neg (38)	Pos (34)	WT
11	ICU	Proven	Lung transplantation	Specific	Pos	Pos	NA	0.4	Neg (42)	Pos (35)	Neg	Neg (38)	
12	ICU	Non-classifiable	AML	Not specific	Neg	NA	0.1	1.6	Neg	Neg	Neg	Neg	
13	ICU	Probable	HL, allogeneic SCT	Specific[g]	Pos	NA	7.2	7.5	Pos (31)	Pos (23)	Pos (31)	Pos (24)	TR_{46}/Y121F/ T289A
14	ICU	Non-classifiable	Liver cirrhosis	Not specific	Neg	NA	0.6	0.3	Neg	Neg	Neg	Neg	
15	ICU	Non-classifiable	Lung transplantation	Not specific	Neg	Neg	0.1	1.2	Pos (31)	Pos (30)	Pos (32)	Pos (32)	WT
16	ICU	Proven	Dermatomyositis	Not specific	Neg	Pos	0.8	5.0	Pos (29)	Pos (27)	Pos (30)	Pos (29)	WT
17	ICU	Non-classifiable	HIV	Not specific	Pos	NA	NA	0.2	Pos (33)	Pos (33)	Neg	Pos (35)	WT
18	ICU	Probable	Dermatomyositis	Specific	Neg	NA	NA	5.9	Neg (36)	Neg (36)	Neg	Neg (40)	
19	ICU	Probable	Dermatomyositis	Specific	Neg	NA	5.2	5.9	Pos (33)	Pos (34)	Pos (35)	Neg (38)	WT

Table 1. Clinical characteristics, radiological findings and results of the galactomannan and AsperGenius® multiplex real-time polymerase chain reaction (PCR) for 22 bronchoalveolar lavage (BAL) samples of patients with proven, probable and non-classifiable invasive aspergillosis (IA). (*continued*)

Patient no.	Clinical setting[a]	IA diagnosis[b]	Underlying disease/ host factor(s)[c]	Radiological findings	BAL or sputum culture[d]	Pathology[e]	AsperGenius® multiplex real-time PCR[f]						
							Galactomannan level		Ct value of *Aspergillus* species PCR		Ct value of *A. fumigatus* PCR		*Cyp51A* PCR
							Serum	BAL	Supernatant	Pellet	Supernatant	Pellet	
20	ICU	Non-classifiable	AML	Not specific	Neg	NA	8.1	0.2	Neg	Neg (37)	Neg	Neg	
21	ICU	Non-classifiable	Vasculitis	Not specific	Pos	NA	NA	6.5	Pos (26)	Pos (24)	Pos (26)	Pos (25)	WT
22	ICU	Non-classifiable	MM	Not specific	Pos	NA	0.3	22.7	Pos (32)	Pos (26)	Pos (34)	Pos (27)	WT

[a] HEM, hematology. ICU, intensive care unit.

[b] IA, invasive aspergillosis.

[c] MM, multiple myeloma. HSCT, hematopoietic stem cell transplantation. AML, acute myeloid leukaemia. APL, acute promyelocytic leukaemia. CLL, chronic lymphatic leukaemia. HL, Hodgkin lymphoma.

[d] Pos, positive. Neg, negative.

[e] NA, not available.

[f] WT, wildtype. Ct, cycle threshold. Ct value < 36 was considered positive and Ct value ≥ 36 negative.

[g] No specific radiological findings in the lung, but specific cerebral findings on magnetic resonance imaging.

The ROC curves for the different groups are shown in figure 1. The diagnostic accuracy as given by the AUC for the hematology group was 0.92 (95% CI 0.79 – 1.00; p-value < 0.001). The AUC was 0.91 (95% CI 0.76 – 1.00; p-value < 0.001) for the ICU group and 0.91 (95% CI 0.81 – 1.00; p-value < 0.001) for the two groups combined. The most optimal Ct value cut-off for the *Aspergillus* species PCR was < 36 for the 37 BAL samples of the hematology patients. Table 2 shows the IA classification related to the Ct < 36 cut-off and table 3 shows the BAL samples according to the gold standard related to the Ct < 36 cut-off. In the hematology group, the Ct < 36 cut-off resulted in a sensitivity, specificity, PPV and NPV of 88.9%, 89.3%, 72.7% and 96.2%, respectively. In the ICU group (n=40), the Ct < 36 cut-off value resulted in a sensitivity, specificity, PPV and NPV of 80.0%, 93.3%, 80.0% and 83.3%, respectively. Therefore, the overall sensitivity, specificity, PPV and NPV was 84.2%, 91.4%, 76.2% and 94.6%, respectively.

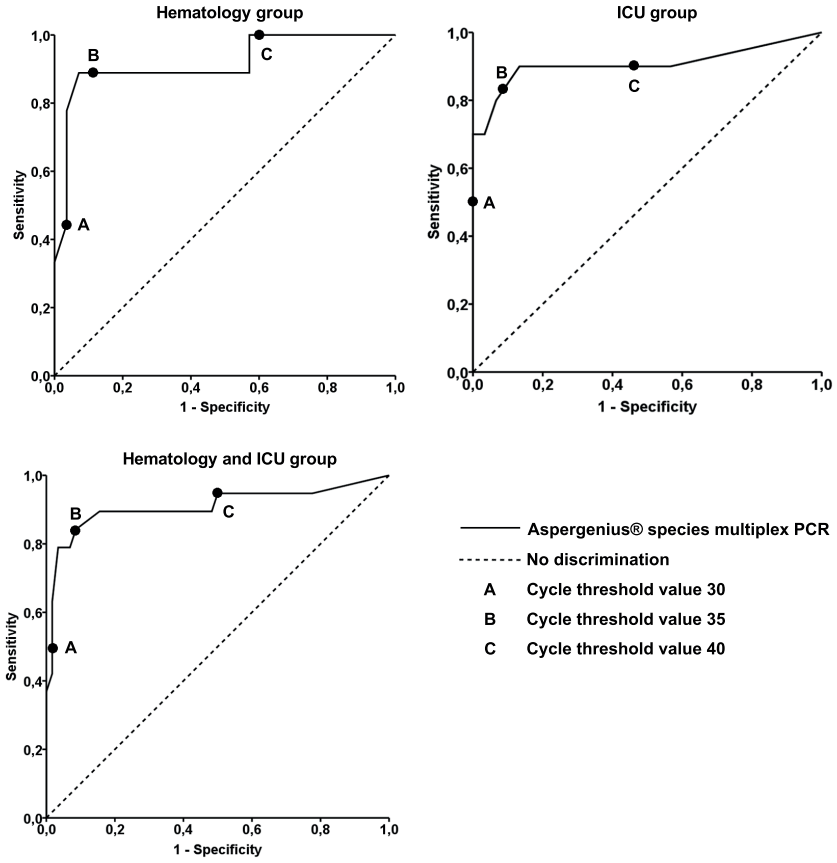

Figure 1. Receiver operator characteristic curves of AsperGenius® species multiplex real-time polymerase chain reaction (PCR) in bronchoalveolar lavage in the hematology, intensive care (ICU) group and combined.

Table 2. Epidemiological classification of invasive aspergillosis (IA) related to cycle threshold (Ct) cut-off of 36.

Classification of IA	Hematology group BAL sample n = 37			ICU group BAL samples n = 40		
	Ct < 36 cut-off	Ct ≥ 36 cut-off	Total	Ct < 36 cut-off	Ct ≥ 36 cut-off	Total
Proven IA	0	0	0	2	0	2
Probable IA	6	0	6	2	1	3
Non-classifiable IA	3	1	4	4	3	7
Possible	2	1	3	0	5	5
No IA	0	24	24	2	21	23

Note: BAL, bronchoalveolar lavage. ICU, intensive care unit.

Table 3. Bronchoalveolar lavage (BAL) samples with positive galactomannan or cultures according cycle threshold (Ct) cut-off of 36.

BAL samples	Hematology group BAL sample n = 37			ICU group BAL samples n = 40		
	Ct cut-off < 36	Ct cut-off ≥ 36	Total	Ct cut-off < 36	Ct cut-off ≥ 36	Total
BAL samples with positive galactomannan ≥ 1.0 and/or with positive culture	8	1	9	8	2	10
BAL samples with negative galactomannan < 1.0 and negative culture	3	25	28	2	28	30

Note: ICU, intensive care unit

In the hematology and ICU patients combined, 19 BAL samples of patients with proven, probable or non-classifiable IA were identified based on the gold standard. From these 19 BAL samples, 16 had a positive *Aspergillus* PCR (15 positive for both the pellet and supernatant, 1 positive for only the pellet). Fourteen of the 16 BAL samples had a positive *A. fumigatus* PCR and the remaining two were *Aspergillus* species. In all 14 positive *A. fumigatus* PCR BAL samples, the *Cyp51A* resistance PCR was successful. Twelve strains were determined as wildtype and two as mutant strains. One sample had a TR_{46}/Y121F/T289A mutation and the other had a TR_{34}/L98H mutation. More information on the melting curves of the mutant strains can be found in the supplement. Both patients of these BAL samples showed clinical failure of voriconazole therapy. The first patient was treated with allogeneic stem cell transplantation for acute myeloid leukaemia. The patient died of culture positive pulmonary and cerebral IA developed during voriconazole prophylaxis (3 days before he died, the single serum voriconazole level was high at 8 mg/L). The second patient developed IA during remission induction chemotherapy for acute myeloid leukaemia. The patient showed an increased serum galactomannan level,

progressive pulmonary infiltrates and pleural effusion (galactomannan levels in pleural fluid were > 2.0) despite 14 days of therapeutic voriconazole serum levels (>4 mg/L, supplement figure S4). Cultures were repeatedly negative. The patient survived after surgical drainage and 8 weeks of liposomal amphotericin-B combined with voriconazole.

DISCUSSION

In this study, the AsperGenius® multiplex real-time PCR showed good performance characteristics for the diagnosis of IA directly on clinical samples in 2 distinct patient populations at risk for this infection. In addition to the fast and correct identification of *A. fumigatus*, this PCR simultaneously differentiated azole susceptible from resistant strains. In contrast, current conventional microbiological tools for susceptibility testing of *A. fumigatus* are rarely helpful because they are time-consuming, not widely available and patients with IA are mostly culture negative. This multiplex real-time PCR assay tackles these problems by enabling diagnosis within hours after a BAL sample is submitted to the laboratory.

In the last decade, the use of galactomannan testing and high-resolution computed tomography of the lungs allowed for an early diagnosis of IA. Together with the availability of voriconazole as the preferred therapy, this resulted in a major decrease in IA-related mortality [3,20]. However, now that azole resistance in *A. fumigatus* is increasing [5-10], the availability of azole resistance testing with a short turn-around time is critical to secure this improved survival in patients with IA. The PCR that was validated in this study can serve this purpose. In accordance with the PCR results, patients can be switched to other non-azole antifungal therapy in an early phase and not when patients clinically deteriorate.

The ROC curves showed that the most optimal Ct value cut-off was 36. This cut-off was comparable to the cut-off described for the 2 *Aspergillus* PCR assays tested by Torelli *et al.* [21]. Moreover, these PCR assays were validated on BAL samples of hematology and ICU patients, the same subset of patients as in the present study. Given these observations, the 36 Ct value cut-off is probably accurate.

In the hematology group, there were one false negative and two false positive *Aspergillus* PCR results when compared with the gold standard. The false negative result was in a BAL sample of a patient with non-classifiable IA (patient no. 5 in table 1). The lung abnormalities could also be a side effect of the chemotherapy that the patient received for her acute promyelocytic leukaemia. It is possible that this patient did not have IA and that the galactomannan of 1.1 in BAL was false positive. The two false positive *Aspergillus* PCR BAL samples were from patients with possible IA. The BAL galactomannan of the first patient was 0.7 and of the second was 0.4. The first patient was treated with

antifungal therapy because there was no alternative diagnosis. In the second patient, a lung biopsy was performed that showed an organizing pneumonia without signs for a fungal infection. As with every diagnostic test, there is no galactomannan cut-off with a 100% diagnostic accuracy. A BAL galactomannan cut-off of 0.5 has an increased sensitivity but a somewhat lower specificity [22]. For PCR validation purpose of this study, we considered a higher specificity more important and therefore we selected the 1.0 galactomannan cut-off as the gold standard.

The PCR assay was performed on DNA extracted from both the pellet and the supernatant of the BAL. The DNA extraction from the pellet is more labour intensive than the extraction of free-circulating DNA from the supernatant. Therefore, it is reassuring that 15 of 16 supernatants were PCR positive. In the remaining patient only the PCR of the DNA extracted from the pellet was positive (Ct value < 36). In this particular patient, the PCR positivity of the pellet was corroborated by a positive sputum culture. Therefore, it may be reasonable and more time efficient to first test the supernatant and only test the pellet if the supernatant is negative in patients with a positive *Aspergillus* culture, a positive galactomannan, or a very high clinical suspicion.

The *Aspergillus* species probe showed cross-reactivity for the *P. chrysogenum* and *R. oryzae*. *P. chrysogenum* is rarely pathogenic in humans. *R. oryzae* can cause comparable symptoms as invasive aspergillosis, but the clinical prevalence is low. Furthermore, for both these species, a 1000 times higher load of DNA was needed to get a Ct-value result of 35 with the Aspergillus species probe. Thus, we believe that these species will not compromise the performance of the PCR.

This study has also some limitations. The validation was performed on readily available leftover BAL fluids from historical patients. At the clinical microbiology laboratory of the Erasmus University Medical Center, all superfluous BAL fluids are stored at -20 ^{o}C for future research purposes. Therefore, no selection bias occurred during storage of the samples. Nevertheless, the results of this study should be confirmed on a larger sample set from different hospitals and ideally prospectively collected across different countries. Another limitation is the fact that only the *Cyp51A* mutations that are included in the PCR, will be detected. As such, this PCR will not replace culture-based sensitivity testing and when this PCR is used, the results should be interpreted in the epidemiological context. Finally, the diagnostic characteristics of every test and in particular the PPV will depend on the background incidence of the population tested. Therefore, the PPV and NPV we describe may be different in other patient populations.

When confirmed in a larger study, this PCR may be incorporated in the EORTC/MSG criteria. The *Aspergillus* PCR could be used in combination with galactomannan testing as it provides information on the *Aspergillus* species involved and azole resistance.

In conclusion, this new multiplex real-time PCR allows for a sensitive and fast detection of *Aspergillus*. Furthermore, it can differentiate wildtype from resistant strains

even on culture negative BAL samples. This enables on-time and targeted therapy in IA-patients.

ACKNOWLEDGEMENT

The authors want to thank Jacques Meis and Ferry Hagen for providing azole-resistant *A. fumigatus* strains.

FUNDING

This work was supported by a grant of the Pieken in de Delta programme from Agentschap NL (PID 102028), the Netherlands.

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SUPPLEMENTARY MATERIAL

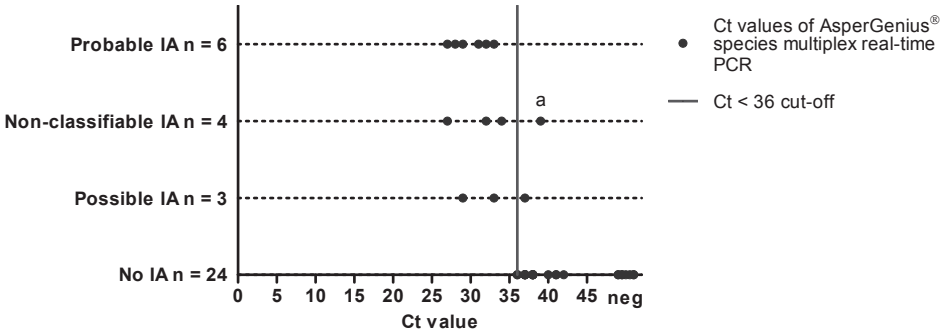

Figure S1. Ct-values of AsperGenius® species multiplex real-time polymerase chain reaction (PCR) in the hematology group.
37 bronchoalveolar lavage samples of hematology patients with probable IA (n=6), with non- classifiable IA (n=4) and no IA (n=24). "a" = patient (no. 5 in table 1) with galactomannan in BAL of 1.1 and in serum of 0.1. The vertical line represents the Ct < 36 cut-off. Ct ≥ 36 are negative (neg) samples.

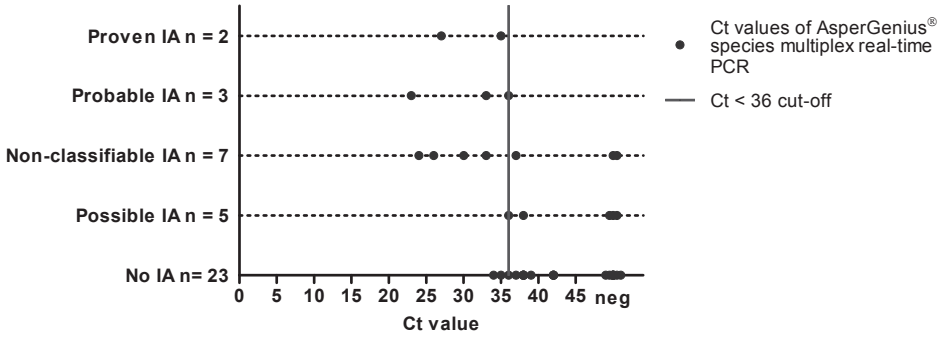

Figure S2. Ct-values of AsperGenius® species multiplex real-time polymerase chain reaction (PCR) in the intensive care (ICU) group.
40 BAL samples of ICU patients with proven (n = 2), probable IA (n=3), non-classifiable IA (n=7), possible IA (n=5) and no IA (n=23). The vertical line represents the Ct < 36 cut-off. Ct ≥ 36 are negative (neg) samples.

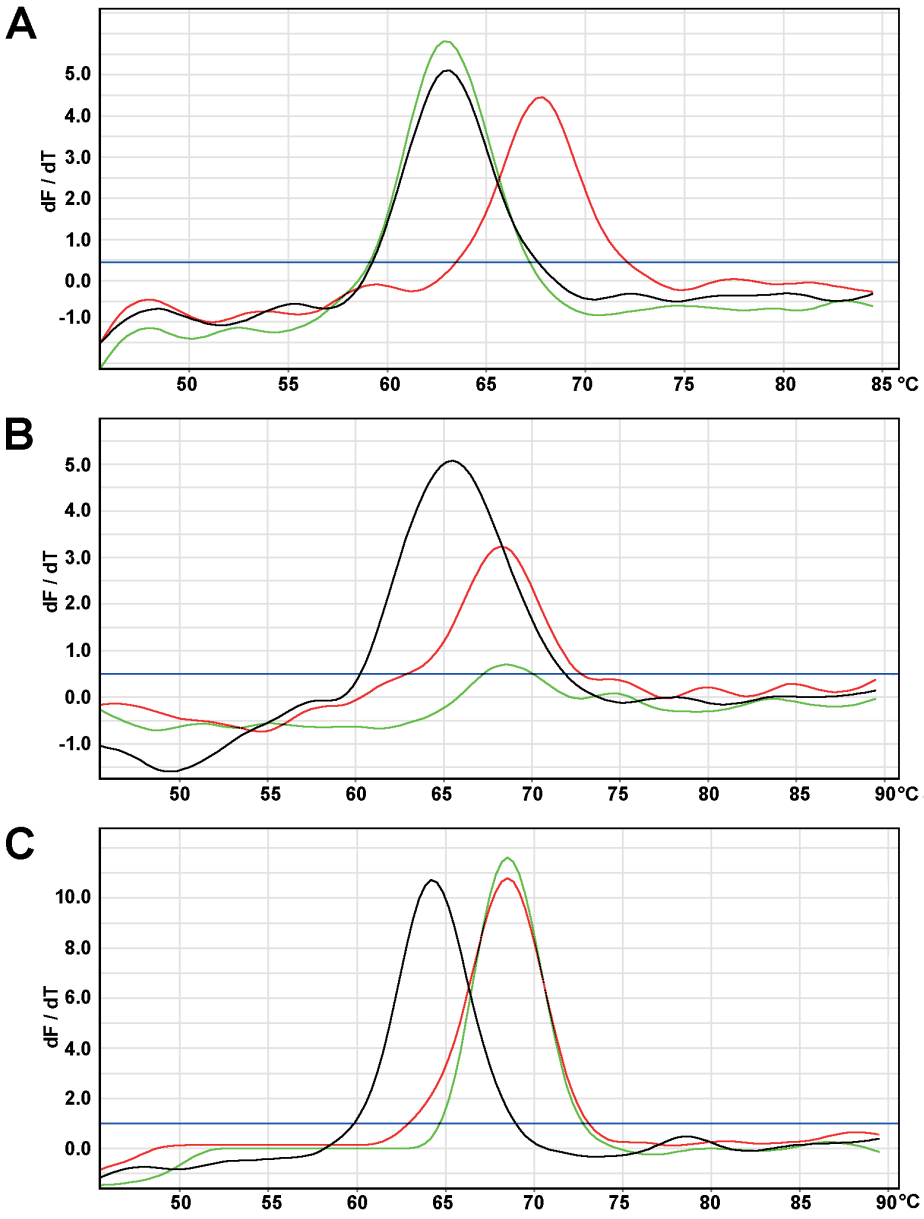

Figure S3. Melting curves of the *A. fumigatus Cyp51A* region.
(A) Patient with an azole susceptible *A. fumigatus* (green) compared to the mutant (red) and wildtype L98 control (black).
(B) Patient infected with an azole resistant *A. fumigatus* TR_{34}/L98H (green) compared to mutant (red) and wildtype TR_{34} control (black).
(C) Patient infected with an azole resistant *A. fumigatus* TR_{46}/Y121/T289 (green) compared to mutant (red) and wildtype T289 control (black).

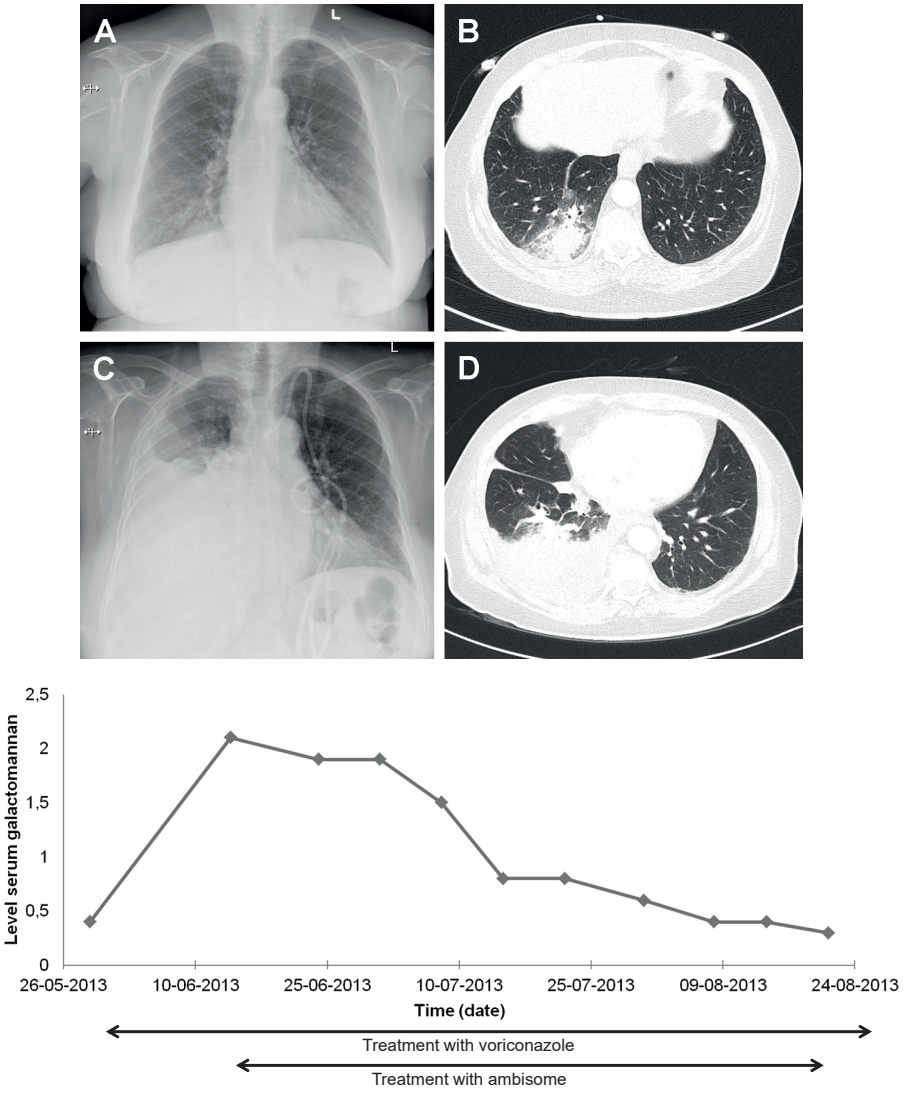

Figure S4. Patient with azole resistant invasive aspergillosis, bronchoalveolar lavage sample showed a TR34/L98H mutation.

Patient was initially treated with voriconazole. After 15 days of treatment, X-ray and computed tomography of the chest showed progressive pulmonary infiltrates and pleural effusion. Liposomal amphotericin-B was added to the treatment. Serum galactomannan eventually became negative after 2 months of voriconazole-liposomal amphotericin-B double therapy.

(A) Chest X-ray before start treatment with voriconazole.

(B) Computed tomography of the chest before start treatment with voriconazole.

(C) Chest X-ray 15 days after start treatment with voriconazole.

(D) Computed tomography of the chest 15 days after start treatment with voriconazole.

(E) Serum galactomannan levels during treatment.

Table S1. Characteristics of the patients of the bronchoalveolar (BAL) samples of the hematology and ICU group.

	Hematology group BAL samples n = 37	ICU group BAL samples n = 40
Mean age in years	58.9 (19.8-83.2)	52.8 (18.3-73.2)
Underlying hematological disease (%)		
Acute myeloid leukaemia	24 (63.2%)	-
Acute lymphatic leukaemia	2 (5.3%)	-
Chronic lymphatic leukaemia	2 (5.3%)	-
Non-Hodgkin Lymphoma	2 (5.3%)	-
Multiple myeloma	4 (10.5%)	-
Myeloproliferative neoplasms	3 (7.9%)	-
Myelodysplastic syndrome	1 (2.6%)	-
Reason ICU admission (%)		
Pneumonia	-	22 (55.0%)
Interstitial lung abnormalities	-	3 (7.5%)
Pulmonary edema	-	3 (7.5%)
Livercirrosis / failure / transplantation	-	2 (5.0%)
Neurological cause	-	3 (7.5%)
Sepsis	-	6 (15.0%)
Multiorgan failure	-	1 (2.5%)
Invasive aspergillosis (%)		
Proven	0	2 (5.0%)
Probable	6 (16.2%)	3 (7.5%)
Possible	3 (8.1%)	5 (12.5%)
Non classifiable	4 (10.8%)	7 (17.5%)
No IA	24 (64.9%)	23 (57.5%)
Treated with following antifungal therapy[a]		
Abelcet	1 (2.7%)	0
Ambisome	6 (16.2%)	6 (15.0%)
Anidulafungin	0	3 (7.5%)
Caspofungin	2 (5.4%)	5 (12.5%)
Fungizone	2 (5.4%)	1 (2.5%)
Micafungin	0	5 (12.5%)
Posaconazol	1 (2.7%)	1 (2.5%)
Voriconazol	22 (59.5%)	18 (45.0%)
No antifungal therapy	14 (37.8%)	18 (45.0%)
Mortality (%)		
At 6 weeks	7 (18.4%)	20 (50.0%)
At 12 weeks	10 (26.3%)	22 (55.0%)
Hospital admission duration (days)		
Hospital stay in total	47.9 (0-388.0)	49.0 (3.0-216.0)
IC stay	-	25.7 (2.0-133.0)

[a] Some patients were treated with more than one antifungal therapy.

Chapter 5.2

PCR-based detection of *A. fumigatus Cyp51A* mutations on bronchoalveolar lavage. A multi-center validation of the AsperGenius assay® in 201 patients with hematological disease suspected for invasive aspergillosis.

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J Antimicrob Chemother 2016;71(12):3528-3535

ABSTRACT

Objectives

In patients with invasive aspergillosis (IA), fungal cultures are mostly negative. Consequently, azole resistance often remains undetected. The AsperGenius® multiplex real-time PCR assay identifies clinically relevant *Aspergillus* species and 4 resistance associated mutations (RAM: TR_{34}/L98H/T289A/Y121F) in the *Cyp51A* gene. This multicenter study evaluated the diagnostic performance of this assay on bronchoalveolar lavage (BAL) fluid and correlated the presence of RAMs with azole treatment failure and mortality.

Methods

Stored BAL samples from patients with hematological diseases suspected for IA were used. BAL samples that were galactomannan/culture positive were considered positive controls for the presence of *Aspergillus*. Azole treatment failure and 6-week mortality was compared in patients with and without RAMs that had received ≥5 days of voriconazole monotherapy.

Results

201 patients contributed each 1 BAL sample, of which 88 were positive controls and 113 negative controls. The optimal cycle threshold cut-off value for the *Aspergillus* species PCR was <38. With this cut-off, the PCR was positive in 74/88 positive controls. The sensitivity, specificity, positive and negative predictive values were 84%, 80%, 76% and 87%, respectively. 32/74 BAL samples were culture negative. Azole treatment failure was observed in 6/8 patients with a RAM compared to 12/45 patients without RAMs (p-value=0.01). Six-week mortality was 2.7 times higher in in patients with RAMs (50.0% versus 18.6%, p-value=0.07).

Conclusion

The AsperGenius® assay had a good diagnostic performance on BAL and differentiated wildtype from *A. fumigatus* with RAMs, also in culture negative BAL samples. Most importantly, detection of RAMs was associated with azole treatment failure.

INTRODUCTION

Invasive aspergillosis (IA) is the most frequent pulmonary mold infection among immunocompromised patients with hematological diseases, and is usually caused by *Aspergillus fumigatus* [1,2]. The triazole voriconazole is currently recommended for first line therapy [3]. However, (pan)azole resistance in *A. fumigatus* has been reported increasingly over the past decade with a prevalence ranging from 1.0% to as high as 20.0% [4-11]. This is worrisome because a study showed that the mortality in culture positive IA caused by an azole resistant strain was 88% [11]. Azole resistance is often caused by mutations in the *Cyp51A* gene that encodes for the lanosterol 14α-demethylase, the target enzyme for azoles. Two mutation patterns in this gene account for a large part of azole resistance mechanisms: TR_{34}/L98H and TR_{46}/T289A/Y121F [6,9-12].

Aspergillus cultures of respiratory specimens are positive in at most 26% of the IA cases [13,14]. Given the low sensitivity of the cultures, most cases are diagnosed indirectly by detection of galactomannan (GM) [13]. However, in the absence of a positive culture, azole resistance remains undetected. Thus, the lack of a fast and readily available azole susceptibility test compromises the initiation of adequate treatment in case of azole resistance. The commercially available AsperGenius® multiplex real-time polymerase chain reaction (PCR) assay consists of 2 PCRs: the species PCR identifies the clinically relevant *Aspergillus* species, and the resistance PCR detects the TR_{34}, L98H, T289A and Y121F resistance associated mutations (RAM) that represent the prevalent mutation combinations TR_{34}/L98H and TR_{46}/T289A/Y121F in the *Cyp51A* gene. In a recent single-center study, the diagnostic performance of the species PCR on bronchoalveolar lavage (BAL) samples of patients with hematological diseases showed a sensitivity, specificity, positive and negative predictive values (PPV, NPV) of 89%, 89%, 73% and 96%, respectively, when a cyclic threshold (Ct) value of <36 was used [15]. Moreover, the resistance PCR was able to detect RAMs in a culture negative patient with IA [15].

The purpose of this retrospective multicenter study was to confirm the diagnostic performance of the AsperGenius® assay in a large population of patients with hematological diseases and to evaluate if the molecular detection of the above-mentioned RAMs correlate with azole treatment failure and mortality.

METHODS

Study design

This retrospective study was performed at 3 Dutch and 2 Belgian hospitals (Erasmus University Medical Center, Leiden University Medical Center, University Medical Center Groningen, Ghent University Hospital and University Hospitals Leuven). The AsperGenius® assay was

performed on 1 milliliter stored leftover BAL samples on which GM (Platelia™ Bio-Rad inc.) and culture had already been performed because of a clinical suspicion of IA. Only BAL samples from patients with hematological diseases were included. Collecting BAL samples ended on 31st of May, 2015. All available leftover BAL samples with a minimum volume of 1 milliliter before this date were obtained. BAL samples were stored at -20 degrees in 4 hospitals and at -80 degrees in 1 hospital. The following information was retrieved from medical files: age, sex, underlying disease, duration of hospitalization, documentation of IA and the antifungal treatment given. In addition, 6 and 12-week mortality was documented. Because this was a retrospective study, no data on the BAL procedure itself was registered.

The study consisted of 2 parts. First, the optimal Ct value and diagnostic performance of the species probe of the PCR were determined. Secondly, treatment failure and 6-week mortality were determined in all patients who had received azole monotherapy for at least 5 days and in whom the resistance PCR successfully discriminated wildtype from *Cyp51A* mutated *A. fumigatus*. Patients with *A. fumigatus* without RAM (=wildtype) were compared to those with *A. fumigatus* containing a RAM. Patients were excluded from the azole treatment failure analysis if (i) a non-*A. fumigatus* or mixed infection was present (e.g. *A. fumigatus* and *A. terreus*), (ii) patients were treated with non-azole therapy or combination therapy, (iii) the antifungal therapy or duration was unknown, or (iv) patients received no therapy. Azole treatment failure was defined as a switch from an azole to any other antifungal drug class. Data of the patient population with hematological diseases of the previous study (n=10) were pooled with the data of the current study for the specific analysis of azole treatment failure and 6-week mortality [15]. The pooling of data was deemed necessary and appropriate because of (i) the rarity of patients infected with RAMs and (ii) the identical methodology and same study site in both studies.

One BAL sample per patient was included in the study. If for a given patient multiple BAL samples were available, the BAL sample of the period with the highest IA classification was selected. In case of multiple BAL samples for a given patient with the same IA classification, 1 BAL sample was randomly selected.

PathoNostics tested the BAL samples blindly and was not involved in the analysis of the results. GMC and BJAR analyzed the data.

Ethics

The medical ethics committees approved the study under the reference numbers MEC-2014-628, P14.337, UC UZG 2014/1217 and S57319. For one Dutch center, local approval was not necessary as approval given by another Dutch medical ethics committee also implied approval for that center. In centers with an opt-out system, all included patients were cross-checked with the list of patients that had objected to the opt-out system. In one center, opt-out forms were sent to the surviving patients to give them the opportunity to refuse the use of their clinical data.

Categorization of BAL samples

BAL samples with a positive GM (≥1.0) and/or a positive *Aspergillus* culture of the BAL, sputum or lung biopsy (<6 days after the date of the BAL) were considered positive controls for the presence of *Aspergillus* in BAL samples. Negative controls were BAL samples with a negative BAL GM in combination with a negative culture from BAL, sputum or biopsy. BAL samples from patients with only a positive serum GM (≥0.5) but a negative BAL GM were considered as negative controls as there was no microbiological evidence of the presence of *Aspergillus* in the BAL sample itself on which the PCR was performed.

Definitions of invasive fungal disease

Patients were categorized as having proven, probable or possible invasive fungal disease according to the revised European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) consensus criteria [16]. In addition, patients with appropriate host criteria and positive microbiological findings but with non-specific radiological features were classified as *non-classifiable* disease. Although this category is not included in the EORTC/MSG definitions, in clinical practice, these patients are treated similarly to those with probable IA given their similar outcome [17].

Processing of BAL samples

One milliliter of the BAL sample was used for DNA-extraction. Samples were processed as described previously [15], with the exception that DNA was extracted from the BAL supernatant and pellet by using the NucliSENS® easyMag® (bioMérieux). The onboard lysis protocol and 50 microliter elution was selected for this purpose before the DNA eluate was used in the AsperGenius® assay. The AsperGenius® assay was performed on the BAL supernatant and pellet separately. A LightCycler 480 II (Roche) was used to perform the AsperGenius® assay. For the determination of the Ct values, the 2nd derivative function of the LightCycler 480 software (v1.5.62) was applied.

AsperGenius® multiplex real-time PCR assay

The AsperGenius® multiplex real-time PCR assay (PathoNostics, Maastricht, the Netherlands) was used to detect *Aspergillus* species and *Cyp51A* gene mutations. The species PCR allows for detection of *A. fumigatus* complex, *A. terreus* and *Aspergillus* species by targeting the 28S rRNA multicopy gene. The *A. fumigatus* probe detects the most relevant species of the *Fumigati* complex: *A. fumigatus, A. lentulus,* and *A. felis*. The *Aspergillus* species probe specifically detects *A. fumigatus, A. terreus, A. flavus* and *A. niger.* An internal control is included to monitor for inhibition or manual handling errors. The resistance PCR targets the single copy *Cyp51A* gene of *A. fumigatus* and detects TR_{34}/L98H/Y121F/T289A mutations to differentiate wildtype from mutant *A. fumigatus* via melting curve analysis.

Each extracted BAL sample was tested in duplicate and a no template control (blank) was included in each run to exclude contamination. A sample was considered positive when one of the duplicates showed increased fluorescence above the threshold. The positive control from the assay was used as a standard for the melting peaks and was tested simultaneously with the BAL samples to determine if the melting peak represents wildtype or *A. fumigatus* with RAM. The resistance PCR was deemed successfully when the supernatant or pellet showed melting peaks for (i) at least 1 of the TR_{34} or L98H, together with (ii) at least 1 of T289A or Y121F resistance markers.

Statistical analysis

The optimal Ct cut-off and diagnostic performance was determined for the species probe of the PCR using the earlier Ct value of the supernatant or pellet. Using the controls as described above, the receiver operator characteristic (ROC) curve and its area under the curve (AUC) were determined (IBM® SPSS® statistics, version 21). The closest to (0,1) point and Youden index were used to further assess the optimal Ct cut-off [18]. The sensitivity, specificity, PPV and NPV were calculated for all BAL samples in total and per hospital. The positive and negative likelihoods were calculated for the most optimal Ct value.

As an additional sensitivity analysis, we determined the ROC curves, AUC and diagnostic performance when using the patients with EORTC/MSG proven, probable IA versus patients without IA. This was thought to be appropriate because the EORTC/MSG criteria are often used for antifungal therapy studies. Because clinicians tend to treat patients with non-classifiable IA in the same way as proven or probable IA [17], we did a second sensitivity analysis in which patients with proven, probable or non-classifiable IA were compared to patients without IA.

For the azole treatment failure and 6-week mortality analysis, the 2-sided Fisher's exact test was used, with a p-value<0.05 considered statistically significant.

RESULTS

In total, 228 BAL samples from 201 patients were available. Samples were obtained between December 2007 and May 2015. No patients refused to have their clinical data used for the purpose of this study. As only 1 BAL per patient was used, 201 BAL samples were available for the analysis. Seven patients with proven, probable or non-classifiable IA were counted as negative controls since there was no evidence of *Aspergillus* in the BAL itself from culture or GM (5 positive serum GM, 1 positive sinus culture and 1 positive lung biopsy culture obtained 16 days after the BAL). The clinical characteristics of the 201 patients are summarized in table 1.

Table 1. Clinical characteristics of the 201 hematology patients who contributed bronchoalveolar lavage (BAL) fluid samples.

	Patients (n = 201)
Age, mean years (range)	56.6 (17.5 – 82.6)
Male gender (n,%)	132 (65.7)
Diagnosis (n,%)	
Acute myeloid leukemia	78 (38.8)
Acute lymphocytic leukemia	16 (8.0)
Chronic lymphocytic leukemia	13 (6.5)
Myelodysplastic syndrome	17 (8.5)
Hodgkin's lymphoma	10 (5.0)
Non-Hodgkin's lymphoma	38 (18.9)
Myeloproliferative disorders	8 (4.0)
Plasma cell disorders	11 (5.5)
Aplastic anemia	3 (1.5)
Other[a]	7 (3.5)
Allogeneic stem cell transplantation (n,%)	82 (40.8)
Invasive aspergillosis (n,%)	
Proven	9 (4.5)
Probable	43 (21.4)
Non-classifiable	43 (21.4)
Possible	32 (15.9)
No IA	74 (36.8)
BAL galactomannan and/or culture positive or negative (n,%)	
Galactomannan and culture positive	28 (13.9)
Galactomannan positive and culture negative	56 (27.9)
Galactomannan negative and culture positive	4 (2.0)
Galactomannan and culture negative	113 (56.2)
Treated with following antifungal therapy (n,%)[b]	
Amphotericin B lipid complex	6 (3.0)
Liposomal amphotericin B	40 (19.9)
Conventional amphotericin B deoxycholate	4 (2.0)
Caspofungin	26 (12.9)
Itraconazole	3 (1.5)
Posaconazole	18 (9.0)
Voriconazole	112 (55.7)
Study anidulafungin versus placebo	3 (1.5)
Study voriconazole versus isavuconazole	3 (1.5)
Study voriconazole versus posaconazole	6 (3.0)
No antifungal therapy	56 (27.9)
Mortality (%) after IA diagnosis	
At 6 weeks	50 (24.9)
At 12 weeks	65 (32.3)
Hospital admission duration (days, range)	
Hospital stay in total	40.7 (2 – 236)

[a] Monoclonal B-cell lymphocytosis, auto-immune hemolytic anemia, sickle cell disease, hemophagocytic lymphohistiocytosis**,** T-cell prolymphocytic leukemia.

[b] There were patients who were treated with more than one antifungal therapy. Some patients were treated within a clinical trial in which the prescribed antifungal therapy was unknown for physicians.

Eighty-eight BAL samples were positive controls, of which 74 (84.1%) were positive for the species PCR (58 positive in supernatant and pellet, 10 only positive in supernatant and 6 only positive in pellet). The species PCR detected 66 *A. fumigatus*, 2 *A. fumigatus* combined with *A. terreus,* 2 *A. terreus* and 4 *A.* species. Thirty-two of these 74 (43.2%) BAL samples were culture negative and only GM positive.

Twenty-three BAL samples from negative controls were species PCR-positive. Five of these 23 BAL samples were from patients with proven, probable or non-classifiable IA (3 diagnosed on positive serum GM, 1 on positive sinus culture, and 1 on positive lung biopsy pathology plus culture). Eleven BAL samples were from patients without IA and 7 from patients with possible IA.

The ROC curve of the species PCR is shown in figure 1. The diagnostics accuracy as given by AUC was 0.890 (CI 95% 0.842–0.939; p-value<0.001). The closest to (0,1) point designated Ct<38 as optimal cut-off, while the Youden index designated Ct<36 as optimal cut-off (table S1). The Ct cut-off of <38 resulted in a sensitivity, specificity, PPV and NPV of 84.09%, 79.65%, 76.29% and 86.54%, respectively. A Ct cut-off of <36 gave values of 70.45%, 95.56%, 92.58% and 80.60%, respectively (table 2). Because a higher sensitivity was preferred over a higher specificity, the Ct value of <38 was chosen for the purpose of subsequent analyses that we performed. The positive likelihood ratio was 4.13 and the negative likelihood ratio 0.20. Table 3 shows the distribution of the BAL samples according to their IA classification. Table S2 shows the diagnostic performance per hospital. As a sensitivity analysis, the diagnostic characteristics were calculated when

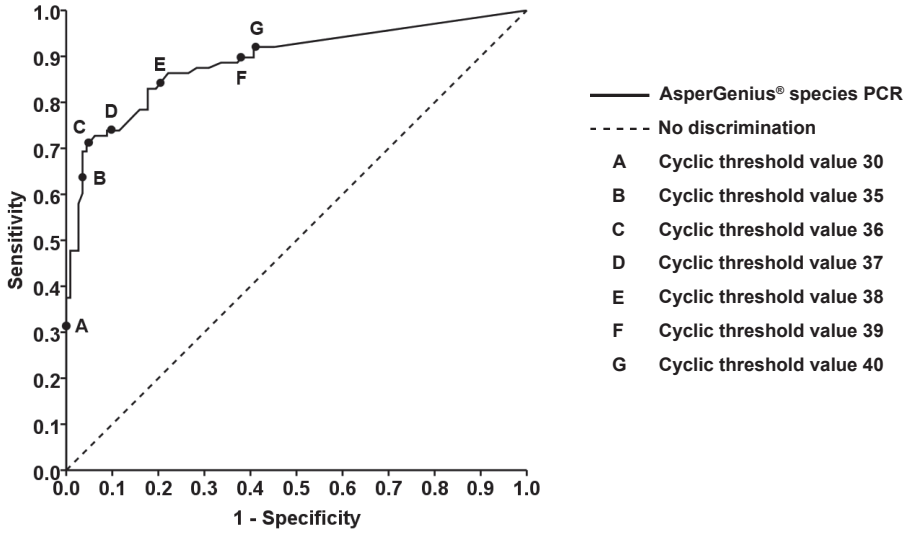

Figure 1. Receiver operator characteristic curve of species probe of the AsperGenius® PCR in the bronchoalveolar lavage fluid samples of the 201 patients with hematological diseases.

patients with proven or probable IA were considered as positive controls and compared to patients without IA. The sensitivity increased to 88% when the <38 Ct cut-off was used. See table 2 and figure S1 for the ROC curves when patients with proven, probable or non-classifiable IA were considered as positive controls.

Table 2. Diagnostic performance of the species probe of the AsperGenius® PCR according to different cycle threshold (Ct) cut-offs and positive/negative controls.

Ct value cut-off of the AsperGenius® species PCR	Diagnostic performance[a]	Positive control versus negative control BAL samples as defined in this study[b] n = 201	Proven, probable or non-classifiable IA versus no IA[c] n = 169	Proven or probable IA versus no IA n = 126
<36	Sensitivity (%)	70.45	68.42	76.92
	Specificity (%)	95.58	98.65	98.65
	PPV (%)	92.54	98.48	97.56
	NPV (%)	80.60	70.87	85.88
<37	Sensitivity (%)	73.86	71.58	78.85
	Specificity (%)	90.27	94.59	94.59
	PPV (%)	85.53	94.44	91.11
	NPV (%)	81.60	72.16	86.42
<38	Sensitivity (%)	84.09	83.16	88.46
	Specificity (%)	79.65	85.14	85.14
	PPV (%)	76.29	87.78	80.70
	NPV (%)	86.54	79.75	91.30
<39	Sensitivity (%)	88.64	87.37	90.38
	Specificity (%)	62.83	72.97	72.97
	PPV (%)	65.00	80.58	70.15
	NPV (%)	87.65	81.82	91.53

[a] PPV, positive predictive value. NPV, negative predictive value.

[b] Bronchoalveolar (BAL) samples with a positive GM (≥1.0) and/or positive culture for *Aspergillus* of BAL, sputum or lung biopsy at most 5 days from date of the BAL were considered positive controls. BAL samples with a negative BAL GM in combination with a negative culture from BAL, sputum or lung biopsy were considered negative controls.

[c] Proven and probable IA was defined according to the revised European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) criteria. Non-classifiable is defined as a patient with EORTC/MSG host and microbiological criteria fulfilled and a pulmonary infiltrate without a halo or air-crescent or well-defined nodule. IA, invasive aspergillosis. No IA was defined as no proven IA, no probable IA, no non-classifiable IA or no possible invasive fungal disease.

Table 3. Distribution of the bronchoalveolar lavage (BAL) samples according to their invasive aspergillosis (IA) classification and species probe of the AsperGenius® PCR using cycle threshold (Ct) value of <38 as cut-off.

Classification of IA	BAL samples n = 201		
	Ct <38	Ct ≥38	Total
Proven	9	0	9
Galactomannan and culture positive	5	0	
Only galactomannan positive	3	0	
Only culture positive	1	0	
Probable	37	6	43
Galactomannan and culture positive	11	0	
Only galactomannan positive	22	6	
Only culture positive	4	0	
Non-classifiable	33	10	43
Galactomannan and culture positive	12	0	
Only galactomannan positive	20	10	
Only culture positive	1	0	
Possible	7	25	32
No IA	11	63	74
Total	97	104	201

Note: galactomannan in BAL and serum. Culture in BAL and elsewhere in the body.

In the 201 BAL samples, the species PCR was positive in 97 BAL samples (figure 2). The resistance PCR was successfully performed in 68/97 BAL samples. Fifty-seven patients had a wildtype *A. fumigatus* and 11 patients had an *A. fumigatus* with RAM (TR_{34}/L98H mutation (n=7), TR_{46}/T289A/Y121F mutation (n=1) and TR_{34}/L98H mutation combined with a wildtype *A. fumigatus* (n=3)). One or two resistance markers were not detected in 9/73 BAL samples (L98H in 4, TR_{34} in 5 and T289A in 2). But based on the detection of the other corresponding resistance marker (e.g. successful amplification of L98H in 4 patients in combination with unsuccessful TR_{34} amplification), a conclusion could be drawn on the presence of *A. fumigatus* wildtype or resistant due to TR_{34}/L98H or TR_{46}/T289A/Y121F.

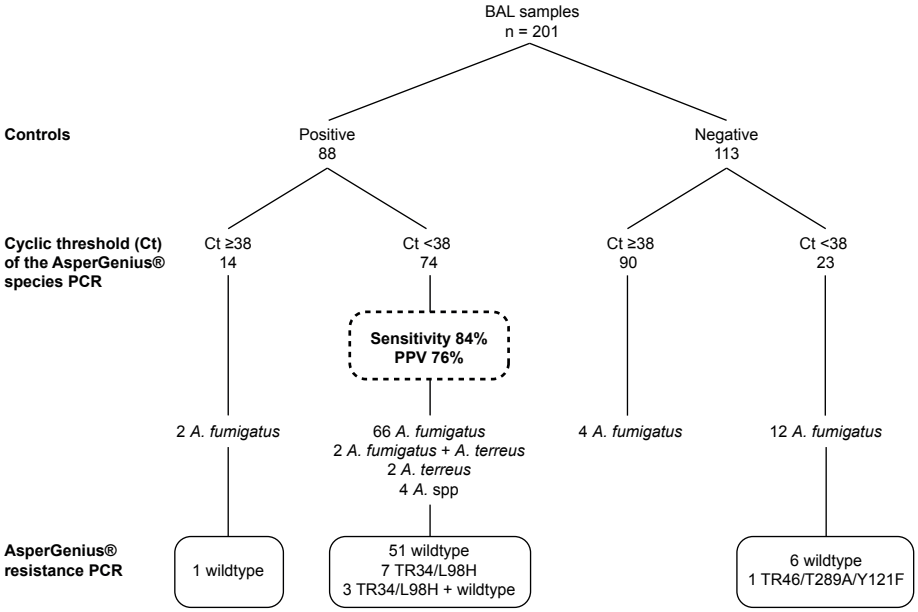

Figure 2. Bronchoalveolar lavage (BAL) samples divided according to positive/negative controls and species probe of the AsperGenius® PCR and resistance PCR.
Two patients had a co-infection with an *A. fumigatus* and *A. terreus*.

For the azole treatment failure and 6-week mortality analysis, resistance data from the 68 patients were included together with 10 patients with hematological diseases from the previous study (8 wildtype, 1 TR_{34}/L98H and 1 TR_{46}/T289A/Y121F).[15] As such, the total group consisted of 78 patients of whom 65 were infected with wildtype *A. fumigatus* and 13 with *A. fumigatus* carrying a RAM. Twenty-five of these 78 patients were excluded from the analysis as described in the methods and figure 3. After exclusion, 45 patients with wildtype *A. fumigatus* remained and 8 with an *A. fumigatus* that contained RAMs. The patients infected with wildtype had 5 proven, 20 probable, 17 non-classifiable IA and 3 possible IA. In patients infected with *A. fumigatus* containing RAM, 4 had probable, 3 had non-classifiable and 1 had possible IA. *A. fumigatus* was cultured in 23/53 patients (3 with and 20 without RAMs). Data on antifungal susceptibility testing were available for 7/23 *A. fumigatus* cultures (5 azole susceptible and 2 azole-resistant) and correlated with the results of the resistance PCR. Azole treatment failure was observed in 12/45 patients with wildtype *A. fumigatus* compared to 6/8 patients with *A. fumigatus* with RAM (p-value=0.01). Six-week mortality was 2.7 times higher in patients with detected RAM (18.6% without versus 50.0%, p-value=0.07).

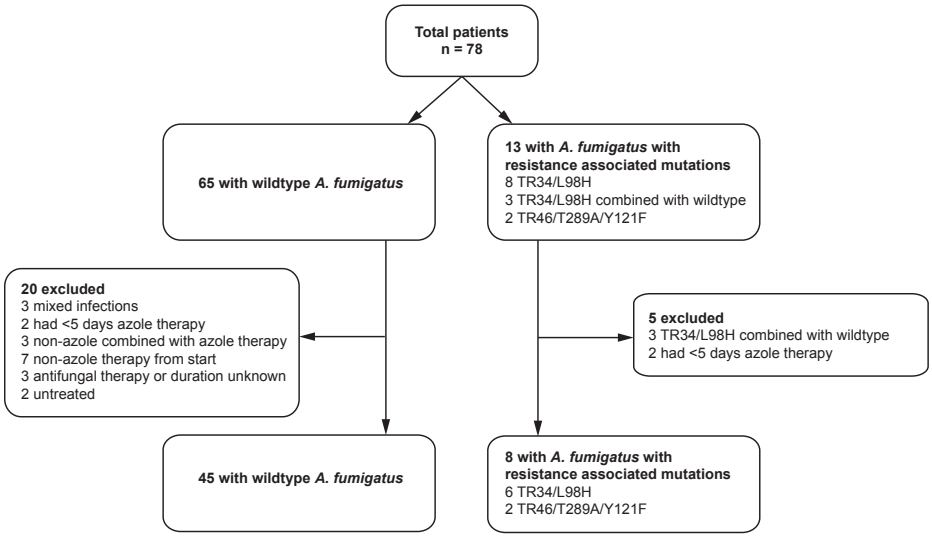

Figure 3. Inclusion for the azole treatment failure and 6-week mortality analysis.

DISCUSSION

This retrospective multicenter study showed that the AsperGenius® species PCR has a good diagnostic performance on BAL samples of patients with hematological diseases. The sensitivity, specificity, PPV and NPV were 84%, 80%, 76% and 87%, respectively, when a Ct cut-off of <38 was used.

The 2 most frequently used statistical methods to analyze a ROC curve are the closest to (0,1) and Youden index. In this study, they led to contradictory results on the most appropriate Ct cut-off values (36 and 38, respectively). Given the important clinical consequences of a missed IA diagnosis, a later Ct cut-off of 38 that results in a better sensitivity may be preferred by the clinician if the resulting loss in specificity is acceptable. Therefore, we favor the use of the Ct cut-off of <38 which is later than the Ct cut-off of <36 that we reported in the previous smaller single center study.[15] The current sensitivity was somewhat lower than in the previous single center study (89%),[15] and may be explained by the fact that a standard volume of 1 milliliter BAL was used in this study versus 1-2 milliliter in the previous study. The lower volume may have decreased the sensitivity. The difference in sensitivity observed *between* the study centers may be explained by differences in the way the BAL is performed in each center. For example, centers may differ in the volume used to perform the BAL, or a bronchoscopist may lavage 2 different parts of the lung but send it in 1 container to the laboratory which may result in a diluted DNA content if *Aspergillus* is present in only 1 part of the lung. Unfortunately, the way the BAL procedure was done in each patient was not registered.

The difference in sensitivity could not be explained by the differences in BAL storage methods. One hospital stored its BAL samples at -80 degrees and had a sensitivity of 75%, while the other 4 hospitals stored at -20 degrees and had a sensitivity of 71% to 100%. The difference could neither be explained by the duration of BAL storage *before* the PCR was performed because 2 hospitals contributed BAL samples from the period 2014 to 2015 and had a different sensitivity of 88% and 100%.

As with all diagnostic tests, a test should be interpreted within the context of the prevalence of the disease. When azole resistance is low in a certain area, it is expected that the PPV of the PCR will probably drop. Based on the positive and negative likelihood ratios of 4.13 and 0.20, respectively, one can determine the post-test probability in a Fagan nomogram to take the prevalence in the patient population into account.

On top of the detection of *Aspergillus*, the AsperGenius® resistance PCR was able to differentiate *A. fumigatus* without a RAM (=wildtype) from RAM-positive *A. fumigatus*, even in culture negative BAL samples. Most importantly, patients infected with RAM-positive *A. fumigatus* failed significantly more often on azole treatment than those infected with a wildtype *A. fumigatus* (75% versus 27%, p-value=0.01). Therefore, this study is the first to show that PCR-detected resistance is clinically relevant. The incidence of azole treatment failure and the 6-week mortality was determined in the pooled data of the current and previous study [15]. The sole reason to pool the data of both studies is the fact that azole resistant IA is still a rare disease with only 13 cases detected in the 83 PCR-positive patients of the 251 patients included both studies. Given the small numbers, a multivariate analysis was not possible to investigate other predictors of azole treatment failure.

To date, the AsperGenius® assay has been studied on serum samples of patients with hematological diseases in which the species PCR had a sensitivity and specificity of 79% and 100%, respectively, when a Ct cut-off of 39 was used [19]. Next to the AsperGenius® assay, other *Aspergillus* PCRs have been tested on BAL samples and sensitivities varied between 38% and 94% [20-22]. Therefore, the sensitivity of the species PCR found in this study is comparable but with the added advantage that RAMs are detected simultaneously. There are other PCRs alike the AsperGenius® assay that detect *Cyp51A* mutations directly on BAL samples [23-25]. Spiess et al. described the detection of *Cyp51A* mutations on 189 clinical samples in their 1st and 2nd studies combined and found the TR_{34}/L98H mutation on 2 BAL samples and 1 cerebral biopsy [23,24]. Zhao et al. found in their study the *Cyp51A* mutations M220 and PL216 which are also associated with azole resistance, on 2/94 BAL samples. These studies along with the current study show that detection of *Cyp51A* mutation on BAL samples is possible, also on culture negative BAL samples. The current study is the first to show that the detection of RAMs is clinically associated with azole treatment failure.

The study has limitations. First, only *Cyp51A* mutations included in the assay can be detected. To date, more than 15 *Cyp51A* gene-mediated resistance mechanisms have been described [26]. The included mutations TR_{34}/L98H and TR_{46}/T298A/Y121F originate from the environment in contrast to *Cyp51A* mutations that are patient acquired after prolonged azole treatment [7,27,28]. The prevalence for the TR_{34}/L98H mutation can account up to 90.2% of the azole resistant *A. fumigatus*, while for the TR_{46}/T289A/Y121F up to 26.9% [6,9-12]. However, the prevalence varies per region. For example, a study from the United Kingdom found only 2 TR_{34}/L98H mutations among the 45 azole-resistant *A. fumigatus* isolates [7]. The assay should therefore be interpreted within the context of the local prevalence of the *Cyp51A* mutations. Besides the *Cyp51A* mutations, non-*Cyp51A* mechanisms that confer azole resistance have been reported [4,6,8-11]. Therefore, PCR testing does not replace culture-based sensitivity testing that should be performed as well. Second, we studied the BAL samples from December 2007 to May 2015. Azole resistance has increased over the past decade [4-11]. Eleven of the 201 (5.5%) patients were infected with an *A. fumigatus* containing a RAM, which may be an underestimation of RAMs in the current population. Lastly, the retrospective nature of the study is another limitation. Antifungal susceptibility testing was not performed routinely in the past. Therefore, the PCR results could only be correlated with the phenotypical resistance in a small portion of the patients. However, in a prospective study, it would be unacceptable to test BAL samples real-time without reporting the detected *Cyp51A* mutations back to the clinician which obviously will lead to a switch from an azole to a non-azole therapy. Therefore, this retrospective study had the advantage that it became possible to report on 8 patients treated with azoles despite the fact that, in retrospect, they had been infected with RAM-positive and therefore azole resistant *A. fumigatus*.

In conclusion, the AsperGenius® assay showed a good diagnostic performance in detecting IA in patients with hematological diseases and the detection of RAM-positive *A. fumigatus* was associated with azole treatment failure, also when patients were culture negative. Therefore, early detection of RAMs by PCR can lead to a prompt adaptation of the antifungal regimen, hopefully contributing to a more favorable outcome of azole resistant *A. fumigatus* in future patients.

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SUPPLEMENTARY MATERIAL

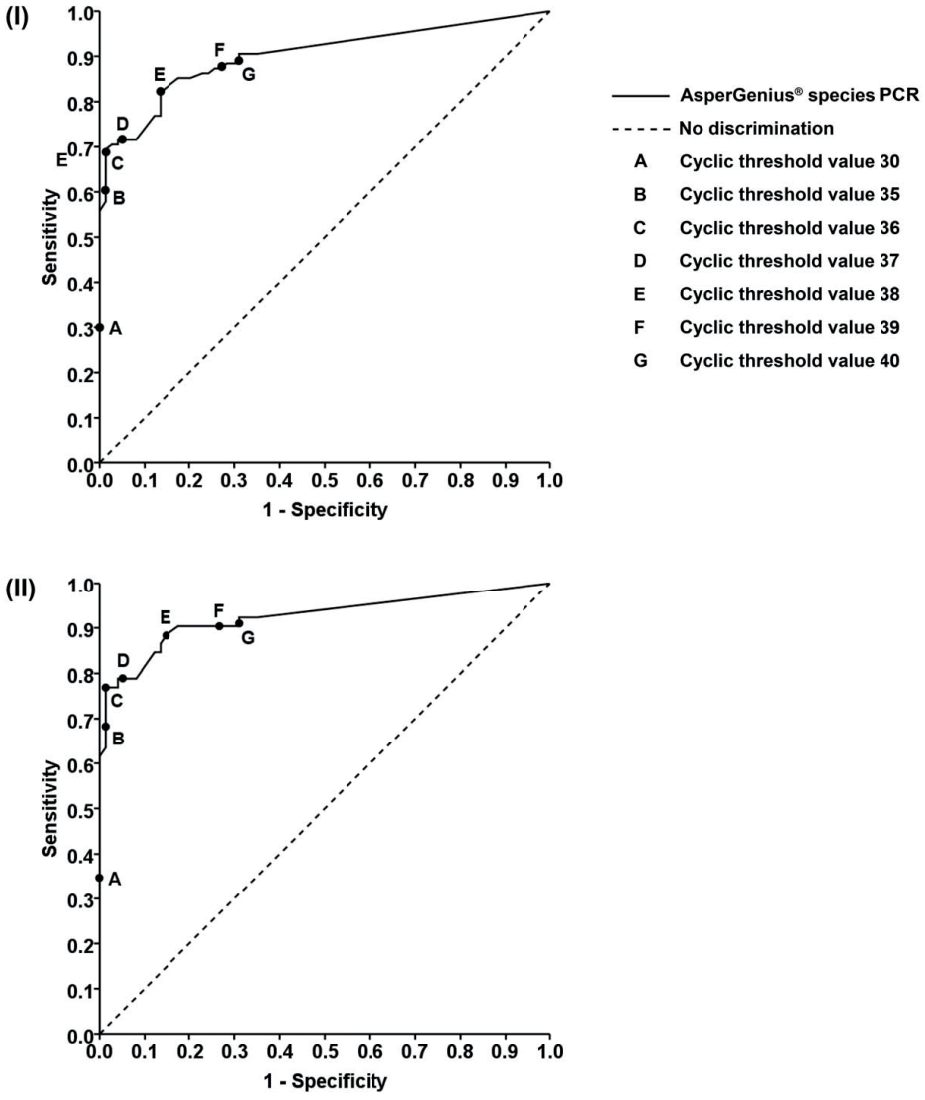

Figure S1. Additional receiver operator characteristic (ROC) curves of species probe of the AsperGenius® PCR in the bronchoalveolar lavage fluid samples.

ROC curve I uses proven (n=9), probable (n=43) and non-classifiable IA (n=43) as positive controls versus no IA (n=74) as negative controls. The diagnostics accuracy as given by area under the curve (AUC) was 0.902 (CI 95% 0.855 – 0.949; p-value<0.001). ROC curve II uses proven and probable IA as positive controls versus no IA as negative controls. The AUC was 0.925 (CI 95% 0.871 – 0.979; p- value<0.001).

Online supplement table S1. Selection of the best cycle threshold (Ct) cut-off using the closest to (0,1) point and Youden index criteria.

Criteria to test optimal cut-off[a]	Positive versus negative BAL samples as defined in this study[b] n = 201	Proven, probable or non-classifiable IA versus no IA[c] n = 169	Proven or probable IA versus no IA n = 126
Closest to (0,1) point			
Ct <36	0.2988	0.3161	0.2312
Ct <37	0.2789	0.2893	0.2183
Ct <38	0.2583	0.2246	0.1881
Ct <39	0.3887	0.2984	0.2869
Youden index			
Ct <36	0.6603	0.6707	0.7557
Ct <37	0.6413	0.6617	0.7344
Ct <38	0.6374	0.6830	0.7360
Ct <39	0.3887	0.6034	0.6335

[a] The closest to (0,1) point selects the lowest distance between a point on a receiver operator curve (ROC) and the point (0,1) as best cut-off. The Youden index selects the maximum vertical distance between the ROC curve and the diagonal or chance line as best cut-off.

[b] Bronchoalveolar (BAL) samples with a positive galactomannan (GM) of ≥1.0 and/or positive culture for *Aspergillus* of BAL, sputum or lung biopsy at most 5 days from date of the BAL were considered gold standard positive. BAL samples with a negative BAL GM in combination with a negative culture from BAL, sputum or lung biopsy were considered gold standard negative.

[c] Proven and probable IA was defined according to the revised European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) criteria. Non-classifiable is defined as a patient with EORTC/MSG host and microbiological criteria fulfilled and a pulmonary infiltrate without a halo or air-crescent or well-defined nodule. IA, invasive aspergillosis. No IA was defined as no proven IA, no probable IA, no non-classifiable IA or no possible invasive fungal disease.

Online supplement table S2. Diagnostic performance of the AsperGenius® species PCR per hospital using cycle threshold value cut-off of <38.

Diagnostic performance[a]	Overall n = 201	Hospital 1 n = 34	Hospital 2 n = 34	Hospital 3 n = 37	Hospital 4 n = 33	Hospital 5 n = 63
Sensitivity (%)	84.09	87.50	90.00	100.00	75.00	70.59
Specificity (%)	79.65	61.11	75.00	82.14	76.47	86.96
PPV (%)	76.29	66.67	96.43	64.29	75.00	66.67
NPV (%)	86.54	84.62	50.00	100.00	76.47	88.89

[a] PPV, positive predictive value. NPV, negative predictive value.

Chapter 6

Interspecies discrimination of *A. fumigatus* and siblings *A. lentulus* and *A. felis* of the *Aspergillus* section *Fumigati* using the AsperGenius® assay

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Diagn Microbiol Infect Dis. 2017;87:247-252

ABSTRACT

Objectives

Aspergillus lentulus and *Aspergillus felis* are sibling species within the *Aspergillus* section *Fumigati* and sporadically cause invasive aspergillosis (IA). They are phenotypically similar but can be reliable identified by molecular techniques. The AsperGenius® assay detects (1) the genus *Aspergillus*, *A. fumigatus* and *A. terreus* at the species level, and (2) *Cyp51A* mutations TR_{34}/L98H and T289A/Y121F of *A. fumigatus*. We report two cases with biopsy proven IA caused by *A. lentulus* and *A. felis,* and the contribution of the AsperGenius® assay in their identification.

Methods

A. lentulus and *A. felis* isolates were obtained from biopsy cultures. Identification was conducted using a sequence-based approach. The AsperGenius® assay was tested on these two isolates and several other control *A. fumigatus, A. lentulus* and *A. felis* species complex isolates.

Results

The AsperGenius® resistance PCR did not detect the TR_{34} target in *A. lentulus* and *A. felis* in contrast to *A. fumigatus.* In addition, melting peaks for L98H and Y121F markers differed between *A. lentulus*, *A. felis* and *A. fumigatus*. The melting curves of the Y121F marker were particularly suitable to discriminate the species. This was confirmed by testing additional isolates (three *A. fumigatus*, six *A. lentulus,* twelve *A. felis* species complex).

Conclusion

The AsperGenius® resistance PCR can be used for accurate interspecies discrimination of *A. fumigatus*, *A. lentulus* and *A. felis* of the *Aspergillus* section *Fumigati.* The latter two species are often azole resistant. The ability to identify them to the species level enables improved management.

INTRODUCTION

Invasive aspergillosis (IA) is mainly caused by *A. fumigatus*, an *Aspergillus* species in the section *Fumigati* [1,2]. The standard therapy for IA is voriconazole [3]. A relatively low mortality is observed when the diagnosis is made early and treatment with voriconazole is initiated promptly [4,5]. However, over the past decade, azole resistance has emerged worldwide and poses a threat as IA with azole resistant *A. fumigatus* is associated with high mortality of 88% [6-8]. Resistance in *A. fumigatus* is often caused by two common mutations (TR_{34}/L98H and TR_{46}/T289A/Y121F) in the *Cyp51A* gene that encodes for lanosterol 14α-demethylase, the target enzyme for azoles [6-8]. In addition to infections caused by azole resistant *A. fumigatus* strains, there are also IA cases caused by species morphologically similar to *A. fumigatus*, so called intrinsic azole resistant 'sibling species' [1,9-13]. These sibling species also belong to the *Aspergillus* section *Fumigati* and can be reliable distinguished from *A. fumigatus* by molecular sequencing.

One of these sibling species was described in 2005 and was named *A. lentulus* because of its slow sporulation as lentulus means 'somewhat slow' in Latin [14]. Subsequently, several reports described patients with IA caused by *A. lentulus* [1,9,11,13,15]. The majority of these reported patients died despite treatment. The *A. lentulus* isolates cultured from these patients had higher minimum inhibitory concentrations (MIC) values for voriconazole, itraconazole, posaconazole, amphotericin-B and caspofungin in comparison to *A. fumigatus*. The intrinsic low susceptibility for azoles of *A. lentulus* can partly be explained by its *Cyp51A* gene. This hypothesis is supported by (i) the observation that *A. lentulus* without a *Cyp51A* gene has significantly lower MIC values for azoles, (ii) *A. fumigatus* transformants harboring the *Cyp51A* gene of *A. lentulus* showed significantly higher MIC values than the *A. fumigatus* wildtype strains [16], (iii) *Saccharomyces cerevisiae* strains expressing the *A. lentulus Cyp51A* gene were significantly less susceptible for azoles than those strains expressing an *A. fumigatus Cyp51A* gene [17].

Another sibling species in the section *Fumigati* that sporadically causes IA is *A. felis*. To date, only two human cases of IA caused by *A. felis* have been reported [10,12]. In both cases, the isolates were initially misidentified as *A. viridinutans* and later identified as *A. felis* [18,19]. These isolates had high MICs to voriconazole and itraconazole, but low MICs to posaconazole and caspofungin, and variable MICs to amphotericin B [10,12]. The resistance mechanism of *A. felis* remains unclear.

The occurrence of IA due to resistant *A. fumigatus* (sibling) species warrants emphasis on prompt identification of these infecting species and their resistance profile. As *in vitro* drug susceptibility testing is often not feasible, as cultures remain negative or sibling species fail to sporulate, molecular techniques are an option. The AsperGenius® multiplex real-time polymerase chain reaction (PCR) assay detects the genus *Aspergillus*, *A. fumigatus* and *A. terreus* to the species level. In addition, it detects the aforementioned

two most common mutation combinations of *A. fumigatus* that are associated with azole resistance. The sensitivity and specificity of the assay ranged from 84.1% to 84.2% and 79.7% to 91.4% in bronchoalveolar (BAL) fluid, and was 78.8% and 100% in serum, respectively [20-22]. Here, we report two patients with proven IA caused by *A. lentulus* and *A. felis* species complex, respectively, and the contribution of the AsperGenius® assay to their identification.

METHODS

The AsperGenius® multiplex real-time PCR assay (PathoNostics, Maastricht, the Netherlands) detects *Aspergillus* species and mutations in the *Cyp51A* gene of *A. fumigatus*. The assay consists of two PCRs: species PCR and resistance PCR. The species PCR identifies the fungus by targeting the 28S rRNA multicopy gene. The *Aspergillus* species probe detects *A. fumigatus*, *A. terreus*, *A. flavus* and *A. niger*. The *A. fumigatus* probe detects relevant *Aspergillus* of the section *Fumigati*. An internal control is included to monitor for inhibition or manual handling errors. The resistance PCR targets the single copy *Cyp51A* gene of *A. fumigatus* and detects the TR_{34}, L98H, Y121F and T289A mutations to differentiate wild-type (WT) from mutant *A. fumigatus* strains via melting curve analysis. The resistance PCR does not likely detect and identify species outside the section *Fumigati* due to differences in the *Cyp51A* gene nucleotide sequence [23].

We first performed the AsperGenius® assay on cultured sibling strains obtained from the two clinical cases to examine i) if the resistance PCR yielded (characteristic) melting curves and ii) if melting curve analysis could be a tool for interspecies discrimination of *Aspergillus* siblings from both WT and mutant *Aspergillus fumigatus*. In addition iii), to assess the precision of the assay, a larger set of strains was tested: six *A. lentulus* isolates and twelve *A. felis* species complex isolates (five *A. felis,* four *A. parafelis* and three *A. pseudofelis*) obtained from the CBS-KNAW Fungal Biodiversity Centre (Utrecht, the Netherlands) and the 3 control *A. fumigatus* isolates (one WT, one TR_{34}/L98H, one TR_{46}/T289A/Y121F). The assay was performed on the isolates in one experiment. Historical data were used to assess the precision of the assay for controls.

The spores of the cultured isolates were dissolved and 50 microliter solution was used for DNA extraction. Samples were processed as BAL pellets (including bead-beating), as described previously [20]. The extracted DNA was tested in duplicate and a template control (blank) was included in each run to exclude contamination. For the species PCR, a sample was considered positive when one of the duplicates showed fluorescence above the threshold. For the resistance PCR, the positive control from the assay was used as a standard for the melting peaks and was tested simultaneously to determine if the

melting peak represents wildtype or *Cyp51A* mutations. A Rotor-Gene Q (Qiagen, Hilden, Germany) instrument was used to perform the AsperGenius® assay.

CASE REPORT 1

A 68-year-old man underwent an upfront allogeneic hematopoietic stem cell transplantation (HSCT) with a matched unrelated donor for myelodysplastic syndrome, refractory anemia with excess of blasts-1. The allogeneic HSCT was complicated by acute graft-versus-host-disease (GvHD) of the skin grade II, for which he was treated with mycophenolic acid 1080 mg twice daily, prednisolone 1 mg/kg daily and cyclosporine of which the dose was based through levels (therapeutic range 250 to 350 µg/l). After recovery of his GvHD, mycophenolic acid was discontinued and prednisolone was tapered to 10 mg daily. Fifty-one days after the allogeneic HSCT, patient was admitted to the hospital because of fever and renal insufficiency. Physical examination showed a temperature of 38.9 °C, but no other abnormalities. Laboratory tests revealed haemoglobin of 4.3 mmol/l, total leucocyte count of 17.3×10^9/l (absolute neutrophil count of 14.6×10^9/l, no further differentiation), C-reactive protein of 181 mg/l and creatinine of 158 µmol/l. Chest X-ray showed bilateral multiple round densities. High resolution computed tomography (HRCT) of the lungs showed multiple round lung lesions with cavitations, suspect for IA. At admission, patient was treated with piperacillin/tazobactam 4000/500 mg four times daily for non-neutropenic fever. The antibiotics were discontinued after 3 days as blood cultures remained negative and HRCT showed radiological signs consistent with pulmonary IA. Voriconazole was started 400 mg twice daily on the first day and 200 mg twice daily afterwards. A BAL was performed, but cultures remained negative. Furthermore, BAL and serum galactomannan were both negative. Consequently, IA was downgraded to 'possible' according to criteria of the European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG) [24]. Despite treatment the patient showed no improvement and continued to have fever. No therapeutic drug monitoring (TDM) of voriconazole was performed. After six days of treatment with voriconazole, antifungal therapy was switched to liposomal amphotericin-B 350 mg daily (5 mg/kg) without success. In the same period, patient developed sepsis with *P. aeruginosa* due to covered perforation based on diverticulitis, for which he received conservative treatment with ceftazidime 2000 mg daily for 17 days. Also, patient had reactivation of Epstein-Barr virus (serum viral load of 6.75×10^3 geq/ml), suspect for post-transplant lymphoproliferative disease (PTLD). As differential diagnosis of the lung abnormalities was invasive fungal disease or PTLD, a biopsy of the lung abnormalities was performed. Lung biopsy showed fibrosis with areas of necrosis with fungal septate hyphae. Culture of the lung biopsy revealed *Aspergillus* section *Fumigati,* and the culture was sent to a

referral laboratory for further identification and sensitivity testing. Patient was switched to treatment with posaconazole 300 mg twice daily for proven IA with *Aspergillus* section *Fumigati*. Concurrently, patient developed progressive renal failure and GvHD of the skin and liver. His clinical condition deteriorated and he died 90 days post-transplantation. Post-mortem, the isolate was identified as *A. lentulus* and had MIC values of 2 µg/ml for amphotericin-B, 2 µg/ml for voriconazole, 0.5 µg/ml for itraconazole and 0.125 µg/ml for posaconazole, respectively. The isolate was deposited in the CBS-KNAW Fungal Biodiversity Centre culture collection (CBS 141342) after confirrmation as *A. lentulus* by sequence analysis of the internal transcribed spacer (ITS) region, and a part of the β-tubulin (*BenA*) and calmodulin (*CaM*) gene (GenBank numbers will follow).

CASE REPORT 2

A 54-year-old man was transferred to our hospital because of neutropenic fever. His medical history revealed relapsed chronic lymphocytic leukaemia (CLL). He had been treated with two regimens of chemotherapy. Initially, he had been treated with fludarabine 40 mg/m^2 and cyclophosphamide 250 mg/m^2 every four weeks, which was discontinued after two cycles because of fludarabine induced pancytopenia. Subsequently, he had been treated with 8 cycles of rituximab 375 mg/m^2, cyclophosphamide 750 mg/m^2, vincristine 2 mg and prednisolone 40 mg/m^2 every three weeks. Shortly after his second-line therapy, patient developed pancytopenia due to refractory CLL. Because initially the pancytopenia presented with only thrombocytopenia, he was treated with prednisolone 1 mg/kg due to suspected autoimmune thrombocytopenia associated with CLL. In the same period, patient was admitted to the general hospital because of polymicrobial sepsis with *Fusobacterium*, *Klebsiella oxytoca* and *Enterococcus faecium*, for which he was treated with cefuroxime 750 mg six times daily, gentamicin 300 mg daily and vancomycin 1000 mg three times daily. In addition, patient was diagnosed with probable IA (lung abnormalities on HRCT and serum galactomannan optical density index of 0.5) for which he was treated with voriconazole 200 mg twice daily. Because of these severe infectious complications, patient was transferred to Erasmus University Medical Center for further treatment of his CLL. On transfer, the prednisolone was already discontinued. Antibiotic treatment for the sepsis and voriconazole were continued. During screening for third-line chemotherapy, patient developed neutropenic fever again. Follow-up HRCT showed that the initial consolidation of the right upper lobe had regressed, but a new pleural mass was found in the left upper lobe. Biopsy of this mass was performed and cultured *Aspergillus.* Because of its poor sporulation, it was not possible to determine the sensitivity and an *A. fumigatus* sibling species was suspected. The isolate was initially identified as *A. viridinutans* by sequencing of the *BenA* gene. As the lung biopsy

culture became positive under voriconazole (no TDM performed), the then unidentified *Aspergillus* species, was considered to be azole-resistant and antifungal therapy was switched to liposomal amphotericin-B 290 mg daily (5 mg/kg) for proven IA. Despite the switch to liposomal amphotericin-B, patient continued to have neutropenic fever and developed dyspnea due to progressive infiltrates. As there were no therapeutic options to treat the patient for his CLL during an active infection and no improvement was observed after switching therapy to liposomal amphotericin-B, treatment was discontinued. Patient died of uncontrolled infection shortly after discontinuing liposomal amphotericin-B. Postmortem, the infecting isolate was re-identified as *A. felis* species complex (CBS 141341) based on sequencing the ITS region, and a part of the *BenA* and *CaM* gene. No MICs were available for this *A. felis* species complex isolate.

RESULTS

The AsperGenius® assay was performed on isolates obtained from lung biopsy in case report 1 and from pleural mass biopsy in case report 2. Both isolates gave positive signals for the *Aspergillus* species and *Aspergillus* section *Fumigati.* First, it was examined if the resistance PCR yielded (characteristic) melting curves (figure 1) and melting temperature (Tm; table 1) values, respectively, for all *Cyp51A* markers of the case isolates in comparison to *A. fumigatus* WT and mutants (TR_{34}/L98H and TR_{46}/T289A/Y121F). The TR_{34} target was not detected in the *A. lentulus* and *A. felis* species complex strains in contrast to WT or mutant *A. fumigatus*. The L98H target showed lower Tm-values for the *A. lentulus* and *A. felis* species complex compared to WT or mutant *A. fumigatus*. Comparable Tm-values for the T289A target were found for *A. fumigatus*, *A. lentulus* and *A. felis* species complex. The Y121F target showed the most different Tm-values for all three sibling species: the Tm variation was 13.5 °C degrees between *A. lentulus* and *A. felis* species complex, 3.0 °C degrees between *A. fumigatus* WT and *A. lentulus,* and 16.5 °C degrees between *A. fumigatus* WT and *A. felis* species complex (table 1). Thus, the absent TR_{34} melting curve with the species specific Y121F melting curve indicates that melting curve analysis is indeed a tool to discriminate *Aspergillus* siblings from both WT and mutant Aspergillus fumigatus.

To assess the precision of the findings the melting curves of six more *A. lentulus* and twelve more *A. felis* species complex strains were analyzed next to the 3 control WT and mutant *A. fumigatus* strains (figure 2). The results of the TR_{34}, T289A and Y121F targets were confirmed for all strains tested. However, the Tm-values of the L98H target were more variable: three different Tm-regions were found for *A. lentulus* and two different Tm-regions for *A. felis* species complex (table 2). Based on the results, again, two targets, i.e. TR_{34} and Y121F can be used to differentiate the siblings *A. lentulus* and *A. felis* species complex from WT and mutant *A. fumigatus*.

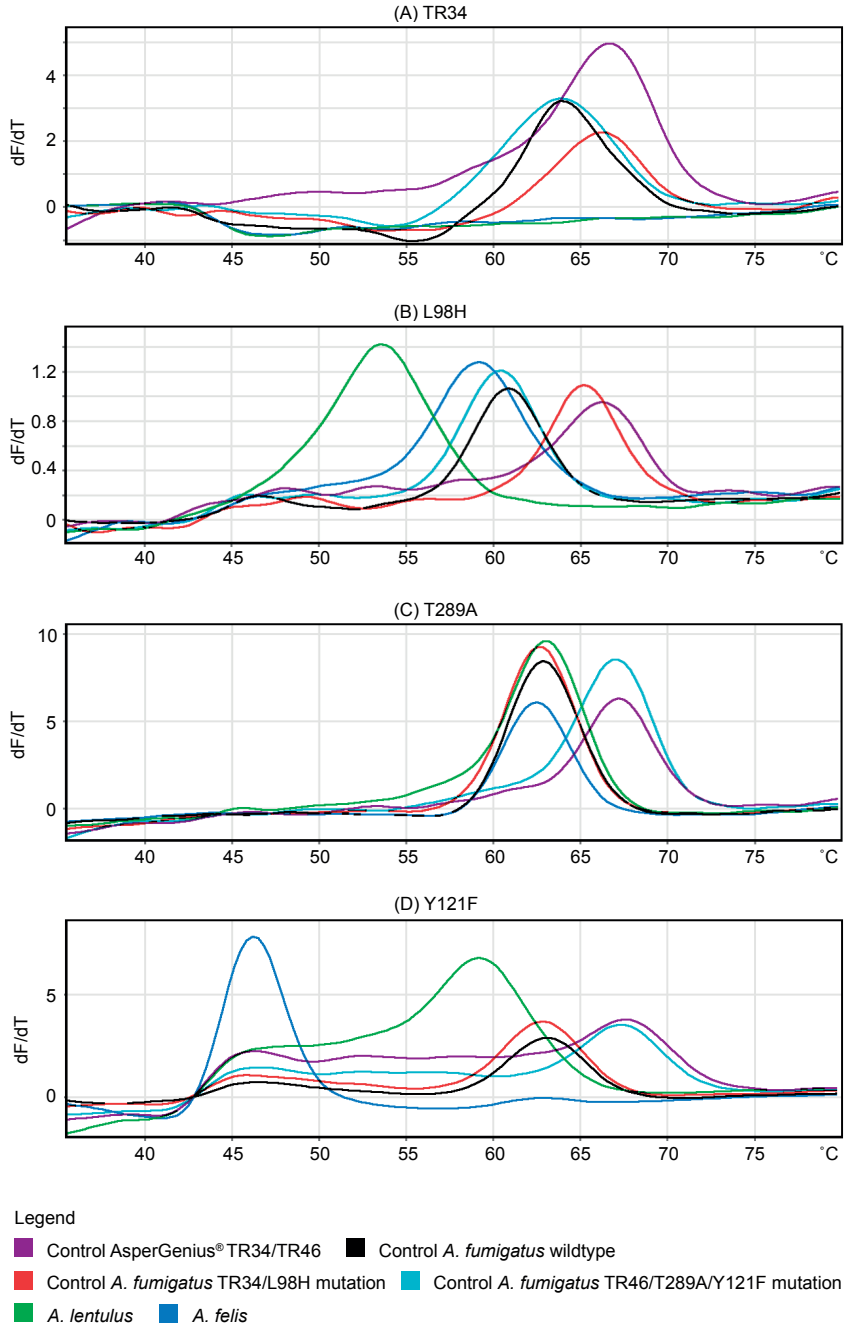

Figure 1. Melting curves of the resistance PCR on lung biopsy cultures of the two sibling species from the cases and control *A. fumigatus* isolates.

Table 1. Melting temperature (Tm) values of the resistance PCR of *A. lentulus*, *A. felis* species complex and wildtype or mutant *A. fumigatus* isolates (controls).

Name	Origen	Tm values			
		TR34	L98H	T289A	Y121F
A. lentulus isolates					
CBS 141342 (Isolate from case 1)	Clinical, NL	ND	54.0	63.0	60.0
CBS 116884	Environmental, Korea	ND	54.0	63.0	59.0
CBS 117887	Clinical, USA	ND	54.0	63.0	59.0
CBS 117886	Clinical, USA	ND	58.0	63.0	59.0
CBS 612.97	Clinical, NL	ND	58.0	63.0	59.0
CBS 117884	Clinical, USA	ND	61.0	63.0	59.5
CBS 117885	Clinical, USA	ND	61.0	63.0	59.0
A. felis species complex isolates					
CBS 141341 (Isolate from case 2)	Clinical, NL	ND	60.5	63.0	46.5
DTO 159-C7 (*A. parafelis*)	Cat, Australia	ND	59.0	62.5	46.0
DTO 176-F1 (*A. parafelis*)	Environmental, DE	ND	59.0	62.5	46.0
CBS 130245^{T} (*A. felis*)	Cat, Australia	ND	60.5	62.5	46.0
CBS 130247 (*A. felis*)	Cat, Australia	ND	60.5	62.5	46.0
CBS 130248 (*A. felis*)	Cat, Australia	ND	60.5	62.5	46.0
CBS 130249 (*A. felis*)	Cat, Australia	ND	60.5	62.5	46.0
CBS 130246 (*A. felis*)	Cat, Australia	ND	60.5	62.5	46.0
CBS 130250 (*A. pseudofelis*)	Cat, UK	ND	60.0	62.5	48.0
DTO 175-H3 (*A. parafelis*)	Environmental, Portugal	ND	60.0	62.5	48.0
CBS 140762^{T} (*A. parafelis*)	Clinical, Spain	ND	60.0	62.5	48.0
CBS 140766 (*A. pseudofelis*)	Clinical, Spain	ND	60.0	62.5	48.0
CBS 140763^{T} (*A. pseudofelis*)	Clinical, Spain	ND	60.0	62.5	48.0
A. fumigatus isolates (controls)					
Wildtype	Clinical, NL	64.5	61.5	63.0	63.0
Mutant TR_{34}/ L98H	Clinical, NL	66.5	65.5	63.0	63.0
Mutant TR_{46}/Y121F/T289A	Clinical, NL	64.5	61.5	67.5	68.0

Note: DE, Germany. NL, the Netherlands. UK, United Kingdom. USA, United States of America. ND, not detected.

To evaluate the precision of the Tm-value differences between wildtype *A. fumigatus, A. lentulus* and *A. felis,* we reviewed Tm-values of nine other clinical WT *A. fumigatus* strains from patients at the Erasmus University Medical Center that we had previously tested with the AsperGenius® assay in the period of January 2015 to June 2016. For the L98H target, the Tm-values of these nine strains ranged from 60.2 to 61.7 °C degrees, which overlapped with the *A. lentulus* and *A. felis.* However, the Tm-values of the Y121F target ranged from 62.5 to 63.3 °C degrees and therefore confirmed that the Tm-values did not overlap with the Tm-values of the *A. lentulus* and *A. felis* species complex isolates.

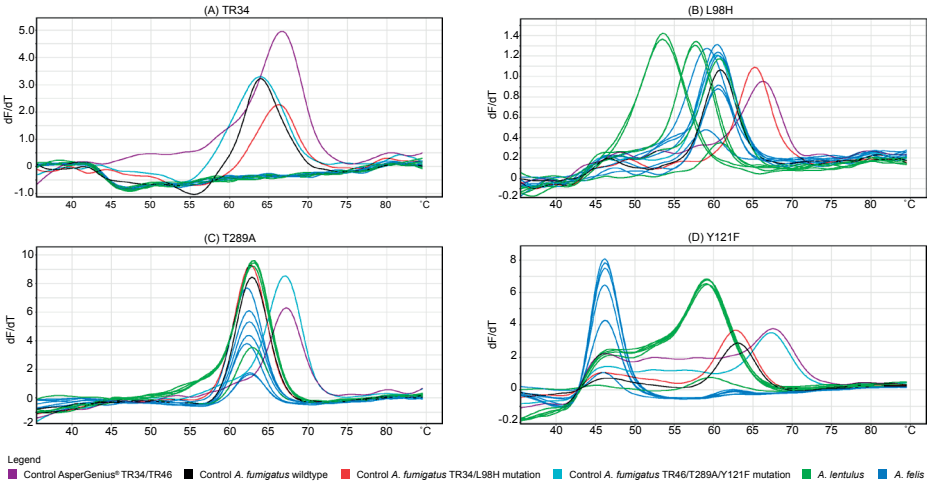

Figure 2. Precision: melting curves of the resistance PCR on the isolates provided by the Fungal Biodiversity Centre.

Table 2. Melting temperature (Tm) and interspecies differentiation of *Aspergilus* section *Fumigati* species with the resistance PCR.

	A. fumigatus WT	*A. fumigatus* TR_{34}/ L98H	*A. fumigatus* TR_{46}/Y121F/ T289A	*A. lentulus*	*A. felis*	Conclusion
L98H	61.5 (historical precision of 60.2 to 61.7)	65.5	61.5	54.0/58.0/61.0	59.0/60.5	Differentiation of *A. fumigatus* TR_{34}/ L98H with other *Fumigati* species. Differentiation of *A. fumigatus* from siblings species not possible.
TR_{34}	64.5 (historical precision of 63.5 to 65.0)	66.5	64.5	ND	ND	Differentiation of *A. fumigatus* TR_{34}/ L98H with *A. fumigatus* WT. Absence of TR_{34} can be indicative for a *Fumigati* species combined with L98H Tm value lower than that of WT (61.5).
T289A	63.0 (historical precision of 62.5 to 63.7)	63.0	67.5	63.0	62.5	Differentiation of *A. fumigatus* TR_{46}/Y121F/T289A with other *Fumigati* species. No differentiation possible of *A. fumigatus* wildtype, *A. fumigatus* TR_{34}/L98H, *A. felis*, *A. lentulus*.
Y121F	63.0 (historical precision of 62.5 to 63.3)	63.0	68.0	59.0	46.0	No differentiation of *A. fumigatus* WT with *A. fumigatus* TR_{34}/L98H. Differentiation possible of all other *Fumigati* species.

Note: ND, not detected. WT, wild-type. Tm, melting temperature.

DISCUSSION

The results reported here showed that the resistance PCR of the AsperGenius® assay not only detects the two most common mutation combinations in the *Cyp51A* gene of the *A. fumigatus* that are associated with azole resistance, but can also be used for interspecies discrimination of the *Aspergillus* section *Fumigati.* Using lung biopsy cultures from two patients with proven IA, we observed that the resistance PCR showed melting curves/Tm-values for *A. lentulus* and *A. felis* species complex that were different from those of *A. fumigatus* WT or *A. fumigatus* with TR_{34}/L98H or TR_{46}/T289A/Y121F mutations. As a result, the resistance PCR could differentiate *A. fumigatus, A. lentulus* and *A. felis* species complex. These results were confirmed on 18 additional isolates.

A. lentulus and *A. felis* species complex belong to *Aspergillus* section *Fumigati* and can sporadically cause IA. They have often high MICs for voriconazole [1,9-13,15]. For *A. lentulus*, its intrinsic low susceptibility for azoles can be explained partly by its *Cyp51A* gene [16,17]. For *A. felis* species complex, its resistance mechanism is unclear. The current results suggest that the *Cyp51A* gene of the *A. felis* is partly alike that of *A. fumigatus* as the resistance PCR gives signals for the L98H, T298A and Y121F targets. Given that voriconazole is the recommended first line therapy for IA [3], a fast identification of *Aspergillus* species that are less susceptible or resistant to voriconazole is important to select the appropriate antifungal therapy. Currently, identification of the two tested sibling species is challenging for two reasons. Firstly, most patients with IA are culture negative and are diagnosed based on a positive galactomannan in serum or BAL fluid [5]. Secondly, if a culture is available, the identification and susceptibility testing of *A. lentulus* and *A. felis* species complex are difficult as they (i) have slow or poor sporulation and (ii) are morphologically similar to *A. fumigatus* except for their inability to grow at 50 °C degrees, and (iii) one or more genes have to be sequenced to confirm their identity [14,19]. Moreover, this identification process is time consuming and often has to be performed in a reference laboratory.

The AsperGenius® assay detects several clinical relevant *Aspergillus* species and differentiates wildtype from azole resistant *A. fumigatus* directly on clinical samples, even in culture negative IA [20,22]. The assay was tested on isolates of two patients with proven IA caused by *A. lentulus* and *A. felis* species complex. As the *A. fumigatus* probe detects these two aspergilli because they are part of the section *Fumigati,* we investigated if the resistance PCR could discriminate the siblings from *A. fumigatus.* This is crucial information, as one would not want to report an isolate as sensitive, when in fact it is resistant to azole treatment (very major error). This would be the case if a sibling would be identified as '*A. fumigatus* by the *A. fumigatus* probe' and sensitive if the resistance PCR of the sibling would be conform the WT melting curve. However, the resistance PCR was able to differentiate between *A. fumigatus, A. lentulus* and *A.*

felis species complex. Not all markers of the resistance PCR were suitable. The T289A marker did not differentiate between the three sibling species. The L98H marker could differentiate WT *A. fumigatus* from TR_{34}/L98H mutant control, but was not able to differentiate *A. lentulus* or *A. felis* species complex. Remarkably, differences were observed in the L98 region between *A. lentulus* strains as well as *A. felis* species complex strains. This is probably caused by small variations in the L98 sequence region. The TR_{34} marker showed no melting curves for *A. lentulus* and *A. felis* species complex in contrast to the wildtype or mutated *A. fumigatus*, which is indicative of a sibling species when the L98H probe produces i) a melting curve and ii) the melting temperature is lower than that of the WT A. fumigatus (63 °C degrees). This is important as the resistance PCR is a single copy PCR and sometimes the TR_{34} might not yield a result whereas the L98H does, which is a known sensitivity issue with low copy numbers. Most importantly, the Y121F marker clearly differentiated *A. lentulus* and *A. felis* species complex from the wildtype as well as the mutant *A. fumigatus*, and was also able to differentiate between *A. lentulus* and *A. felis* species complex. In other words, the resistance PCR can aid in preventing to report a very major error (a sibling species as WT i.e. sensitive to azoles).

The case and control *A. fumigatus, A. lentulus* and *A. felis* species complex strains were tested in one experiment. To assess the precision of this 3 °C degrees difference of the Tm-values from *A. fumigatus* and *A. lentulus*, we reviewed historical data and no overlap was observed for the Y121F. Therefore, when both the melting curve analysis of the TR_{34} and Y121F markers are analyzed, *A. lentulus* and *A. felis* species complex could be differentiated from the *A. fumigatus* WT and *A. fumigatus* with TR_{34}/L98H or TR_{46}/T289A/Y121F azole resistance combinations.

In 2014, Sugui et al. published the results of a phylogenetic analysis on 19 isolates from the section *Fumigati*, of which nine were *A. felis* or *A. viridinutans* [25]. Within these nine isolates, three novel species were distinguished and named *A. pseudofelis*, *A. parafelis* and *A. pseudoviridinutans*. These novel species showed increased MIC values to itraconazole and voriconazole, which is similar to *A. felis* [10,12,25]. The AsperGenius® assay was tested on five *A. felis*, four *A. parafelis* and three *A. pseudofelis* strains. Due to the uncertainty of the taxonomy and no clinical consequences regarding antifungal therapy, we preferred to refer the strains as *A. felis* species complex.

There are a few limitations. First, the resistance PCR detects and identifies polymorphisms in the *Cyp51A* gene as these polymorphisms result in different melting curves. However, susceptibility testing on isolates should be done if possible, to confirm the exact phenotype as well. However, culture-based susceptibility testing is time consuming and often impossible with poorly or non-sporulating species like *A. lentulus* or *A. felis* species complex. Secondly, the resistance PCR was not tested on other siblings in the *Fumigati* section. To date, the *Aspergillus* section *Fumigati* has up to at least 63 species, of which 17 have been reported to be opportunistic pathogens to humans and/or other

mammals [26]. Except for the *A. fumigatus,* the other 16 pathogenic *Aspergillus* species, of which *A. lentulus* and *A. felis* species complex are among the more 'frequent', are described in case reports or as a very small part of collections of clinical isolates [26]. We were unable to test if more sibling species of the section *Fumigati* would have the same melting curves or Tm-values as found in the sibling species in this current report. Further research using the AsperGenius® assay and other sibling species is therefore needed.

We conclude that the AsperGenius® assay accurately detects and differentiates *A. fumigatus* WT, *A. fumigatus* TR_{34}/L98H and *A. fumigatus* TR_{46}/T289A/Y121F from the sibling species *A. lentulus* and *A. felis* species complex using the azole resistance targets TR_{34} and Y121F. *A. lentulus* and *A. felis* species complex are often azole resistant. Retrospectively, the promptly identification of these two sibling species on species/complex level could have enabled improved management in the two cases.

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Chapter 7

General discussion and summary

INTRODUCTION

Invasive aspergillosis (IA) is a life threatening opportunistic infection that is usually caused by *A. fumigatus* [1,2]. It is the most common invasive fungal disease (IFD) in immunocompromised patients with an underlying hematological disease and is associated with a substantial increase in morbidity, mortality as well as health care costs [3-5]. Ever since voriconazole has been shown to be the superior treatment for this infection [6], it has become the cornerstone of IA therapy [7]. However, in 2002 azole resistance was reported for the first time in Dutch patients infected with *A. fumigatus* and its prevalence has continued to increase from that moment on [8,9]. The prompt diagnosis and appropriate treatment of an infection with an azole-resistant *A. fumigatus* is difficult [10,11]. A better insight into the different aspects of IA (diagnostics, outcome but also prevention) will facilitate its optimal management. In this thesis several studies on the epidemiology, prevention and diagnosis of IA are described with a particular focus on high-risk patients with an underlying hematological disease. The current chapter summarizes and discusses the main findings in **paragraph 2**. Recommendations for future research are given in **paragraph 3**.

MAIN FINDINGS AND DISCUSSION

Invasive aspergillosis and allogeneic hematopoietic stem cell transplantation

IA is a common IFD in recipients of allogeneic hematopoietic stem cell transplantation (alloHSCT) [1,12-22]. The reported incidences vary from 2.3% up to 15.0% and the mortality is substantial in this particular patient group [12-18,20]. Primary prophylaxis with voriconazole or posaconazole significantly reduces the incidence of IA in alloHSCT recipients and is recommended in those receiving corticosteroids and/or other therapies for graft-versus-host disease (GVHD) [7,23,24]. However, apart from GVHD, many other risk factors for post-transplant IA have been described, such as older age, neutropenia, non-related donor, reactivation of cytomegalovirus (CMV) and a history of pre-transplant IFD [12-16,19,20,22,25,26]. Moreover, IA is most frequently observed in the first year post-transplantation, but a substantial part of the recipients develops IA later [12,16,17]. Therefore, the best timing and duration of primary prophylaxis can be difficult to decide. Recognizing risk factors may help in selecting those patients that are at highest risk and therefore benefit most of primary prophylaxis. We conducted a retrospective study to determine the incidence, outcome and risk factors of IA in alloHSCT recipients. All patients who underwent their first alloHSCT in the Erasmus University Medical Center between 2004 and 2014, were included. IA was classified according to the European Organization for the treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria

[27]. In addition, patients with a positive microbiological criterium but with non-specific radiological findings were defined as having non-classifiable IA. These patients were included, as clinicians tend to treat them similar to patients with probable IA.

A total of 663 patients received their alloHSCT in the indicated period. We found an incidence of proven, probable, non-classifiable IA of 13.0% within the 12-months post-transplantation and 15.5% in the entire follow-up time. All-cause mortality was significantly higher in patients with than without IA. An increase in the incidence of IA was observed in 2012 and 2013. This may be explained by different reasons. First, a long-lasting demolition and renovation of a neighboring hospital building was initiated from 2011 onwards at the site where all alloHSCT recipients were transplanted and followed after alloHSCT. Hospital constructions and demolitions have been associated with increased IA in patients with underlying hematological disease [28,29]. Second, azole resistance has emerged in the Netherlands over the past 15 years [9,11]. In our cohort, 6 recipients had developed probable or non-classifiable IA due to azole-resistant *A. fumigatus.* As fungal cultures often remain negative and because antifungal susceptibility testing was not a standard procedure in the first half of the observation period, the actual number of patients infected with azole-resistant *A. fumigatus* may have been higher. As primary prophylaxis with triazoles does not prevent azole-resistant IA, the emergence of azole resistance may have contributed to the increased incidence in IA as well.

To study the risk factors, we performed a nested case-control study within the total cohort. Ninety-nine patients with proven, probable and non-classifiable IA (cases) were compared to 198 patients without IA (controls). A backward logistic regression showed that the risk factors for developing IA were neutropenia, lymphocytopenia, pulmonary comorbidity, CMV reactivation, renal impairment and the use of systemic corticosteroids. Also, a higher dose of corticosteroids was associated with a higher risk of IA, suggesting a dose-effect relationship. Most of these risk factors were also found by others [13,16,22,25,26]. However, this study is the first to identify renal impairment as an independent risk factor for IA [12,21,25]. Remarkably, and in contrast to some other studies, pre-transplant IA and GVHD was eliminated among others in the logistic regression analysis [12-14,16,19,20,22,25]. Perhaps pre-transplant IA was not found to be risk factor, because patients with pre-transplant IA are often treated with secondary prophylaxis during alloHSCT. Also, selection bias may partially explain this finding because a subset of patients in need for an alloHSCT who develop IA may no longer be eligible for the transplantation. As for GVHD, patients with acute or chronic GVHD are almost always treated with systemic corticosteroids. Consequently, these variables are highly associated. In an additional univariate analysis, acute and chronic GVHD were analyzed as one variable and found to be a risk factor. However, any form of GVHD was eliminated when added to our current model, presumably because of the strong and dose dependent effect of corticosteroids. Our results do not reject the hypothesis that GVHD *in se* is a

risk factor, but with the current study design, it was not possible to disentangle to what extent the presence of GVHD and corticosteroid therapy are independent risk factors for IA.

In conclusion, 15.5% of the patients develops IA post-transplantation and these patients have a worse outcome than those patients without IA. Although the majority of the patients develop IA in the first-year post-transplantation, there is a continued risk on developing IA after the first year as well. The risk factors that we have identified for IA should help clinicians deciding to start or to discontinue antifungal prophylaxis in the appropriate patient at the correct time. In the Erasmus University Medical Center, primary prophylaxis was formerly prescribed to alloHSCT recipients with GVHD who were treated with second line anti-GVHD treatment. Based on the results of this study, primary prophylaxis was extended to all alloHSCT recipients who are treated with high-dose corticosteroids. In the view of the increasing azole resistance, continued assessment of IA in this patient group is needed to monitor the effect of primary prophylaxis with triazoles.

Inhalation of liposomal amphotericin-B to prevent invasive pulmonary aspergillosis

Chemotherapy-induced prolonged neutropenia can be complicated by invasive pulmonary aspergillosis (IPA). In 2008, a randomized controlled trial (RCT) showed that liposomal amphotericin-B (L-AmB) inhalation twice a week prevented IPA in patients with acute myeloid leukemia (AML) [30]. This resulted in a BI recommendation for the use of this intervention in European Conference on Infections in Leukaemia (ECIL) guidelines [31]. After the RCT, prophylaxis with L-AmB inhalation became the standard of care for patients with AML undergoing remission induction chemotherapy at Erasmus University Medical Center in 2009. Nonetheless, it is difficult to predict how interventions work in real-life outside the context of a RCT. In **chapter 3**, we describe a prospective population-based study in which we establish the external validity of the RCT outcomes in a real-life AML patient population. In total, 127 consecutive AML patients prophylactically inhaled L-AmB during their 1st and 2nd remission induction chemotherapy; 108 patients treated for AML at the same sites from 2005 to 2008 served as controls. A significant decrease in probable and proven IPA was observed in the L-AmB inhalation group (L-AmB group 9.5% vs control group 23.4%, p=0.0064). Systemic antifungal therapy given at any time during the entire AML therapy decreased from 52.8% to 29.9%. Apart from its efficacy, the use of L-AmB inhalation was also cost saving in regard to diagnostics and voriconazole therapy (minus 1816 euro/patient). These cost savings more than compensated for the costs related to L-AmB inhalation itself (1292 euro/patient). No L-AmB inhalation related serious adverse events were observed.

Apart from the current study and the randomized study by Rijnders et al., a third randomized controlled study on the use of amphotericin-B (AmB) inhalation for the prevention of IPA in patients with an underlying hematological disease has been published [30,32]. Schwartz et al. evaluated the effectiveness of conventional AmB inhalations in 382 patients who were neutropenic for at least 10 days after chemotherapy or autologous stem cell transplantation. No difference was found in the IPA incidence or mortality between the inhalation group and no inhalation group. This may be explained by the fact that conventional AmB was used. Conventional AmB can impair the function of the pulmonary surfactant in contrast to L-AmB that exhibits a surfactant-like function [33]. Moreover, another inhalation system was used in this study which could also affect the efficacy of the inhaled medication.

L-AmB inhalation was already the standard of care in the Erasmus University Medical Center when the current study was initiated. Therefore, we compared patients inhaling L-AmB to historical control patients to evaluate the efficacy and cost-effectiveness in real-time. Considering that medicine evolves quickly, a time-related and diagnostic bias might occur when controls are not from the same calendar year as cases. However, no considerable changes occurred in the chemotherapeutic agents that were used for treating AML and the diagnostic procedures remained unchanged throughout the study. Hence, it is unlikely that a time-related or diagnostic bias has occurred.

In an unselected AML patient group, L-AmB inhalation resulted in a statistically significant and clinically relevant decrease in IPA that was also cost saving. With the current study results available together with the clinical trial data from 2008, we think it is time to move forward and study the use of L-AmB inhalation in other patients at high risk for IPA, in particular in patients with serious GVHD. Moreover, it is time that L-AmB inhalation is used outside the Erasmus University Medical Center as this approach does not suffer from a decreased efficacy in the context of triazole resistance problem in the Netherlands. Preventing IA will avoid the need for expensive combination antifungal therapy for patients with IA. Indeed, as a result of the increasing prevalence of azole-resistant *A. fumigatus*, combination therapy is now recommended in the SWAB guideline on invasieve fungal infections (2017) when triazole resistance cannot be excluded by culture or *Cyp51A* PCR testing [34].

Galactomannan antigen testing on cerebrospinal fluid

Cerebral aspergillosis (CA) is a rare and often fatal fungal disease (IFD) [4,35]. The diagnosis is challenging as radiological findings are non-specific and cerebrospinal fluid (CSF) cultures are only positive in less than one-third of the cases [36,37]. Moreover, obtaining brain tissue for histopathological examination, the diagnostic gold standard, is frequently not feasible because of the risk of complications in patients with an underlying hematological disease who are often trombocytopenic. We postulated that testing

cerebrospinal fluid (CSF) for the presence of galactomannan (GM) may help in diagnosing cerebral aspergillosis (CA) [27,36,38,39]. Although GM antigen detection is one of the microbiological criteria in the EORTC/MSG criteria, the test has never been validated properly [27]. In **chapter 4**, we described the findings on the diagnostic performance of CSF GM antigen. The CSF GM levels were compared at different cut-offs in patients with probable and proven CA to those without CA. The revised IA and CA definitions of the EORTC/MSG were used but excluding the to-be-validated-test (=CSF GM) as a microbiological EORTC/MSG criterion. The study population consisted of 44 patients (4 proven CA, 13 probable CA and 27 no CA). When a cut-off of 0.5 to 2.0 was used, GM detection in CSF had a good diagnostic performance with a sensitivity, specificity, positive and negative predictive values were 88.2%, 96.3%, 93.8% and 92.9%, respectively.

To analyze the diagnostic performance of a diagnostic test, a gold standard needs to be chosen to allow for a comparison of the old and new diagnostic test. For GM antigen testing on CSF, the gold standard would be a positive cerebral biopsy to definitely prove the presence of CA. However, cerebral biopsies are not often performed. Hence, we chose to exclude CSF GM from the EORTC/MSG criteria to allow for the evaluation of CSF GM testing. CA was diagnosed in two steps: first, we examined if patients had proven or probable IA outside the cerebrum. Subsequently, these patient with proven or probable IA were diagnosed with CA if they also had suspected cerebral lesions. This was deemed necessary because otherwise patients with an isolated serum GM ≥0.5 and a focal cerebral lesion (e.g. cerebral infarction) but without any other evidence of IA elsewhere would fit the probable CA definition. Using this approach, a good diagnostic performance was found with the GM cut-off of 0.5 to 2.0.

The CSF GM antigen test is included in the revised EORTC/MSG [27]. However, so far only 2 studies have reported on GM antigen testing in CSF in a total of 10 patients with CA [38,39]. Viscoli et al. measured GM on CSF from 5 patients with proven and probable CA, and found a significantly higher GM level in patients with CA compared to patients without CA [39]. Similarly, Kami et al. found that the GM antigen test was positive in 4 of the 5 CSF of patients with proven CA and negative in all patients without CA [38]. Finally, Antinori et al. reviewed the literature on *Aspergillus* meningitis, which is not the same as CA, and found that CSF GM was performed in 15 of the 93 cases [36]. The median CSF GM was 6.58 with a range of 2.2 to 578. The sensitivity was 86.7%.

Retrospective studies have the limitation that only those data that are readily available can be analyzed which may reduce the population size and study power. Unfortunately, this was also a limitation in our current study. GM antigen had been tested on CSF of 157 patients admitted to the hematology, internal medicine and pediatric departments of 2 university hospitals in the period 2004 to 2014. Over half of the patients had to be excluded because not all data that were needed for the analysis were available. This resulted in a considerable reduction in size of the study population. Despite of that,

the current study population (n=44) was the largest study ever published on this topic and consisted of data over 10 years of 2 large academic hospitals.

The results of our study on 44 patients show clearly that the first step in the diagnostic approach to a patient in whom CA is considered, should be CSF GM antigen testing. If this test is positive, the diagnosis is almost certain and treatment should be initiated promptly. On the other hand, a negative CSF GM test makes the diagnosis of CA very unlikely.

Diagnosis of azole-resistant Aspergillus fumigatus

Over the past 15 years, azole resistance in *A. fumigatus* has emerged worldwide [10,11]. This is a worrisome development as small case series have reported a very high mortality in IA caused by azole-resistant *A. fumigatus* [40,41]. Moreover, the diagnosis of azole-resistant *A. fumigatus* is challenging as (i) cultures become positive only in about one-quarter of the IA cases, and (ii) conventional microbiological testing for azole susceptibility is not widely available and is time consuming [42,43]. The lack of a fast and readily available susceptibility testing method in particular for patients in whom cultures remain negative hampers the prompt initiation of adequate therapy in patients infected with an azole-resistant *A. fumigatus*. A potential solution to this problem may be the use of an *Aspergillus* polymerase chain reaction (PCR) assay that can detect the presence of *Aspergillus* DNA and can identify the *Aspergillus* to the species level. In addition, the detection of *Aspergillus* DNA may allow for the detection of mutations that confer resistance to azoles. This method may also be used on patient samples of which fungal cultures remain negative. Such a PCR was investigated in **chapter 5.1 and 5.2**. We described the results of two studies on the AsperGenius® multiplex real-time PCR, a commercially available *Aspergillus* assay that detects several *Aspergillus* species as well as the two most common mutation combinations in *A. fumigatus* that are associated with azole resistance in the Netherlands. In a retrospective single-center study (**chapter 5.1**), the optimal cycle threshold (Ct) and diagnostic value of the aforementioned assay was used on 77 stored BAL samples of patients with an underlying hematological disease or admitted to the intensive care unit (ICU). BAL samples that were GM (≥1.0) and/or culture positive were considered positive controls for the presence of *Aspergillus*. Negative controls were BAL samples with a negative GM in combination with negative culture. We found that the optimal Ct for the presence of *Aspergillus* was <36. Using this Ct value, we found a good diagnostic performance with an overall sensitivity, specificity, positive and negative predictive values (PPV, NPV) of 84.2%, 91.4%, 76.2% and 94.6%, respectively. Moreover, we also identified two patients with IA caused by *A. fumigatus* with TR_{34}/L98H and TR_{46}/T298A/Y121F mutation combinations and who both failed azole therapy.

Subsequently, a multicenter study was performed to confirm the optimal Ct value and diagnostic performance of the AsperGenius® assay in a larger population of patients with underlying hematological diseases (**chapter 5.2**). In this multicenter study, we were also able to evaluate if the molecular detection of the resistance associated mutations (RAM) correlated with azole treatment failure and mortality. In 201 BAL samples of patients with an underlying hematological disease, the receiver operator character (ROC) curve was analyzed according to two different methods to select the optimal Ct value. These two methods led to contradictory results as to the most appropriate Ct cut-off values (36 and 38, respectively). Given the important clinical consequences of a missed IA diagnosis, a higher sensitivity was preferred. Therefore, the later Ct value cut-off of 38 was chosen over 36 that was reported in the previous study and was used for the subsequent analyses. Using the Ct cut-off of <38, a sensitivity, specificity, PPV and NPV of 84.1%, 79.7%, 76.3% and 86.5% was found. This sensitivity was somewhat lower than the sensitivity of 89% that was found in the single center study at a Ct cut-off of <36 in the hematology group. This difference may be explained by the fact that a standard volume of 1 milliliter BAL was used in multicenter study versus 1-2 milliliter in the single center study. The lower volume may have decreased the sensitivity. Also, we observed some differences in the sensitivity *between* the five study centers, which may be explained by differences in the way the BAL is performed in each center. For example, protocols may differ concerning the volume used to perform the BAL, or a bronchoscopist may lavage two different parts of the lung but send it in one container to the laboratory which may result in a diluted DNA content if *Aspergillus* is present in only one part of the lung. Unfortunately, the way the BAL procedure was done in each patient was not registered. The differences *between* the study centers could not be explained by difference in methods or duration in BAL storage.

For the evaluation of the diagnostic performance of the AsperGenius® assay, BAL samples with a positive GM (≥1.0) and/or a positive *Aspergillus* culture of the BAL, sputum or lung biopsy were considered positives. Negative controls were BAL samples with a negative BAL GM in combination with a negative culture. Consequently, BAL samples of patients with specific radiological findings and positive serum GM, but negative BAL GM, were categorized as negatives. This categorization was chosen in favor of the EORTC/MSG criteria to ensure that there was evidence of *Aspergillus* in the BAL samples themselves. However, the EORTC/MSG criteria are often used as criteria for the presence of IA in antifungal studies, and therefore two extra sensitivity analyses were performed using the EORTC/MSG criteria as the diagnostic criteria. Moreover, clinicians tend to treat patients with non-specific radiological findings and positive GM in the same way as proven and probable IA. Therefore, these patients were also included in the sensitivity analyses and described as having 'non-classifiable IA'. Patients without IA were compared to i) patients with proven or probable IA and ii) patients with proven, probable or non-

classifiable IA. When using a Ct value of <38 as the cut-off for positivity, the sensitivity was 88% and 83%, respectively.

In the next step, we analyzed if the detection of RAMs predicted azole treatment failure and 6-week mortality. Patients infected with a RAM-positive *A. fumigatus* failed indeed significantly more often during azole treatment than those infected with a wildtype *A. fumigatus* (75% versus 27%; p-value = 0.01). Also, 6-week mortality was 2.7 times higher in patients with RAMs (50.0% versus 18.6%; p-value = 0.07). Therefore, this study was the first to show that azole resistance detected by PCR is an important clinical finding. This observation led to the implementation of this resistance PCR as standard diagnostic test in clinical practice at the Erasmus University Medical Center. When a patient with an underlying hematological disease is suspected for IA and a BAL is performed, the AsperGenius® assay is performed together with a culture and GM antigen level measurement in the BAL sample.

When using the test, it should be noted that the assay detects the two most common mutation combinations TR_{34}/L98H and TR_{46}/T289A/Y121F that confer pan-azole resistance in the Netherlands [9]. However, the prevalence of these two mutation combinations varies per region [41,44-49]. Moreover, *Cyp51A* mutations and non-*Cyp51A* mutations have been described and are not detected by the assay [41,44,45,47-49]. Therefore, conventional *Aspergillus* cultures with antifungal susceptibility testing are still necessarily to detect phenotypic resistance caused by other mechanisms. The assay should therefore be used in addition to conventional *Aspergillus* cultures and not as replacement. Also, clinicians should realize that the documentation of wildtype *Cyp51A* with the Aspergenius® assay makes the presence of an infection with an azole-resistant *Aspergilllus* much less likely but does not exclude its presence with 100% certainty.

The retrospective design of this study is a limitation. For the diagnosis of azole-resistant *A. fumigatus,* antifungal testing is preferred to confirm its phenotypical resistance. Unfortunately, susceptibility testing was not done routinely in the past and therefore the results of the AsperGenius® assay could not be correlated with the phenotypical resistance in a subset of the patients. However, it would not have been possible to study the azole treatment failure and 6-week mortality in a prospective study as it would have been ethically unacceptable to test BAL samples in real-time without reporting the detected RAMs to the clinician. Hence, the current design made it possible to report on 8 patients treated with azoles despite the fact that, in retrospect, they had been infected with RAM-positive and therefore azole resistant *A. fumigatus*.

To conclude, the Aspergenius® assay had a good diagnostic performance in BAL samples and detects azole resistance caused by the two most common *Cyp51A* mutation patterns, even in culture negative BAL samples. Morerover, we demonstrated that PCR-based resistance testing predicts azole treatment failure. Early detection of PCR-based resistance can lead to a prompt adaptation of the antifungal regimen and hopefully con-

tributing to a more favorable outcome of azole resistant *A. fumigatus* in future patients Therefore, this test should be implemented as the standard diagnostic test in countries were the prevalence of azole resistance is 5% and certainly when it exceeds 10%. In 2016, 12.9% of the *A. fumigatus* stains cultured from patients in the Netherlands were azole resistant [9]. Therefore, antifungal therapy should be guided by resistance testing just like this is the standard procedure in all Dutch hospitals for bacterial infections and antibiotic therapy. PCR-based resistant testing should be performed simultaneously next to the fungal culture in all centers that treat hematology patients at high risk for IA.

Azole-resistant Aspergillus fumigatus sibling species

The AsperGenius® assay detects several *Aspergillus species* and the *A. fumigatus Cyp51A* mutations TR_{34}, L98H, T289A and Y121F that are associated with azole resistance [11,41,50]. In **chapter 6**, we evaluated its contribution in identifying *A. lentulus* and *A. felis,* two rare but intrinsically azole-resistant sibling species within the *Aspergillus* section *Fumigati* [51-53]. Identification of these species with conventional culture techniques is difficult and time-consuming. The assay was tested on (i) two *A. lentulus* and *A. felis* strains obtained from biopsy proven invasive aspergillosis and (ii) control *A. fumigatus* (n=3), *A. lentulus* (n=6) and *A. felis* species complex (n=12) strains. The PCR melting curve analysis for L98H and Y121F markers differed and those of the Y121F marker were particularly suitable to discriminate the three species.

A. fumigatus has up to 63 sibling species in its family, of which 17 have been described to cause disease in humans [54]. Some of these sibling species, also called cryptic species, are intrinsically azole resistant and are difficult to distinguish from *A. fumigatus* sensu stricto by culture [51-53,55-58]. The AsperGenius® assay was investigated on *A. lentulus* and one *A. felis* strain that were cultured from patients with biopsy proven IA. We showed that it is possible to distinguish *A. fumigatus*, *A. lentulus* and *A. felis* from each other by using melting analysis of the PCR assay. This is important to avoid a major error in which the test would recognize the azole-resistant species as being azole sensitive. However, as sibling species are very rare, we were not able to investigate if the assay could also distinguish other sibling species.

In conclusion, the assay can be used to rapidly discriminate *A. fumigatus* from *A. lentulus* and *A. felis*, which is important as the latter two *Aspergillus* species are intrinsically resistant for triazoles.

FUTURE DIRECTIONS

The studies in this thesis have contributed to new insights into invasive aspergillosis in patients with an underlying hematological disease. This chapter will elaborate on some of the opportunities for future research based on these new insights.

Over the past decade, azole resistance has emerged and its prevalence continues to rise in the Netherlands [9]. Azoles are the first choice treatment for patients with an IA because their use is clearly associated with a reduction in mortality [6,7]. However, in regions where azole resistance is higher than 10%, therapy that includes L-AmB or an echinocandin have been recommended [11,59]. The updated SWAB guideline on the management of invasieve fungal infections has now a chapter on azole-resistant *A. fumigatus* and recommends to start with combination therapy that includes an azole plus a second antifungal drug (L-AmB or an echinocandin) in critically ill patients suspected of having IA. In less ill patients, azole monotherapy can still be given provided that the documentation of azole susceptibility or resistance is pursued by performing BAL sampling for culture and resistance PCR testing [34].

It has been shown that azole-resistant *A. fumigatus* with TR_{34}/L98H and TR_{46}/T289A/Y121F originate from the environment [60]. Conidia of azole-resistant *A. fumigatus* are inhaled together with the conidia of azole sensitive *A. fumigatus*, and subsequently both can cause IA simultaneously in a single patient. Prophylaxis with azoles may therefore lead to outgrowth of the azole-resistant *A. fumigatus* in a high-risk patient. Whether the rising prevalence of azole resistance should change antifungal prophylaxis strategies is currently uncertain, but it seems obvious that the efficacy of azole prophylaxis will decrease in the context of an increasing prevalence of azole resistance in *A. fumigatus* in the environment. We showed that prophylaxis with L-AmB inhalation is effective in preventing IA in AML patients undergoing remission induction therapy **(chapter 3)**. It is inhaled two times a week and is a good alternative for azole prophylaxis for patients who are hospitalized or for those who visit the outpatient clinic twice a week or more. However, L-AmB inhalation is not registered for the use as prophylaxis and the implementation is logistically somewhat challenging. However, this should in no way withhold hematology units from implementing L-AmB inhalation in the Dutch landscape of an ever increasing prevalence of azole resistance given the dramatic consequences of an infection with an azole-resistant *A. fumigatus* for the individual patient. As far as we know the Erasmus University Medical Center is still the only center in the Netherlands in which this strategy has become the standard of care prophylactic regime for patients with AML undergoing remission induction therapy. Now that it has been clearly demonstrated that L-AmB inhalation effectively protects neutropenic AML patients from developing IA, further research should focus on other high-risk patients such as recipients of an alloHSCT.

The AsperGenius® assay was shown to be of value in the diagnosis of IA caused by azole resistant *A. fumigatus* **(chapter 5)**. There are still questions regarding the diagnostic performance of the assay. In the single-center and multicenter study, all the BAL samples were tested in a single laboratory. It would be of interest to see how the assay would perform in different laboratories. In the multicenter study, we showed that the detection of RAMs is associated with azole treatment failure. The question that now needs to be answered urgently is if the early detection of RAMs and the immediate switch to non-azole therapy will be able to improve the very high mortality observed in patients infected with an azole-resistant *A. fumigatus*. Ideally, this question should be investigated in a prospective multicenter study. Also, in a prospective study, susceptibility testing could be performed and correlated with the results of the AsperGenius® assay. Such a prospective multicenter study was designed in 2016 and started in 2017, and was called the AzoRMan study, AZOle Resistance MANagement study (NCT number NCT03121235) [61]. In this study, a standard diagnostic and therapeutic protocol for IA is used in patients with an underlying hematological disease who present with new pulmonary infiltrate suspicious for IA (figure 1). This protocol is comparable to the current approach that was already used in the Erasmus Medical University Center. The primary objectives of this study are:

1. To improve the outcome of patients infected with azole-resistant *A. fumigatus* by early detection of RAMs and with this, earlier initiation of the most appropriate therapy;
2. To monitor the prevalence of IA due to *A. fumigatus* strains carrying the TR_{34}/L98H or TR_{46}/T289A/Y121F RAMs in the Netherlands, in particular in culture negative patients.

All 8 university hospitals in the Netherlands have agreed to participate, and 60 of the 280 patients were included as of March 2018.

To date, the AsperGenius® assay has been studied on *Aspergillus* cultures, BAL samples, serum and plasma. The assay had a sensitivity and specificity of 79% and 100% in serum, and 80% and 77.8% in plasma, respectively for the detection of *Aspergillus* [62,63]. However, successful amplification of the RAMs in serum was achieved only in 50% of the proven and probable cases [62]. Therefore, future studies are needed with the aim of improving the sensitivity of the resistance PCR. In 2017, the ARPOS study (Azole Resistance PCR Prospective Optimisation-study) was designed and will study the diagnostic performance of *Aspergillus* PCRs on serum or plasma of immunocompro-

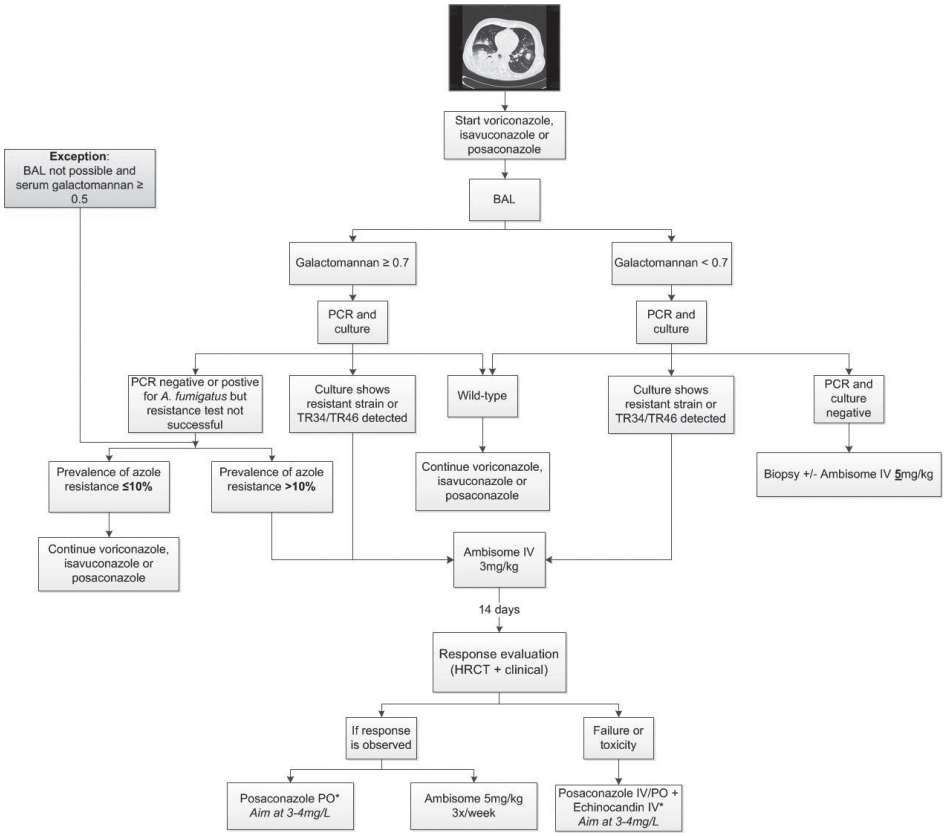

Figure 1. Diagnostic and therapeutic protocol used in the AzoRMan study.
A minimum inhibitory concentration of >2 in culture susceptibility testing means that the *A. fumigatus* is voriconazole resistant according to the method of the Clinical Laboratory Standards Institute. TR_{34}/TR_{46} means that a resistant associated mutation (RAM) is detected via the AsperGenius® PCR and the *Aspergillus* isolate is considered as resistant. PCR and culture negatieve means that the AsperGenius® PCR and culture was not successful, and therefore no conclusions can be drawn about the absence or presence of azole resistance. Galactomannan <0.7 is considered negative for BAL fluid samples. * Posaconazole should not be considered an option if the posaconazole MIC is >1 mg/L. This flowchart was taken from the protocol of the AzoRMan study.

mised patients with an underlying hematological disease. The primary objectives of this study are:

1. To determine the best serum or plasma volume to generate the best diagnostic performance;
2. To determine whether serum or plasma is the best medium of *Aspergillus* DNA extraction;
3. To compare different available *Aspergillus* species PCRs and to determine which PCR is most suitable to be used in a future large prospective clinical study that will evaluate the use of serum or plasma *Aspergillus* PCR for the early diagnosis of IA;

4. To determine what the percentage is of conclusive resistance PCR results that show the presence or absence of of the TR_{34}/L98H and TR_{46}/Y121F/T289A mutation combinations when the PCR is performed on serum/plasma of patients with probable or proven IA (based on the result obtained from PCR testing on BAL fluid).

The study will start recruiting patients in the Erasmus University Medical Center and 2 Belgian hospitals in 2018.

FINAL REMARKS

The studies described in this thesis resulted in valuable new insights into the epidemiology, prevention and diagnostics of IA in high-risk patients with an underlying hematological disease. Several risk factors were found for developing IA in alloHSCT recipients. Prophylactic L-AmB inhalation was shown to decrease IPA in patients with AML and was also cost-effective. The added value of GM antigen testing on CSF was shown when the diagnosis of CA is to be confirmed. The AsperGenius® assay was proven to have a good diagnostic performance for the detection of IA in BAL samples and predicted azole treatment failure when PCR-based resistance was detected. Finally, this assay was also able to discriminate *A. fumigatus* from two intrinsically azole-resistant sibling species and therefore avoiding a major error.

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Chapter 8

Nederlandse samenvatting

INTRODUCTIE

Invasieve aspergillose is een levensbedreigende opportunistische schimmelinfectie. Dit betekent dat deze infectie in principe voorkomt bij patiënten met een langdurig ernstig verlaagde weerstand en niet bij mensen met een normale weerstand. De ziekte wordt veroorzaakt door de schimmel *Aspergillus*. Er zijn verschillende *Aspergillus* soorten, maar de meest voorkomende verwekker voor invasieve aspergillose is de *Aspergillus fumigatus*. *Aspergillus fumigatus* is alom aanwezig in het milieu en wordt door mensen ingeademd. Bij gezonde mensen ruimt het immuunsysteem de *Aspergillus fumigatus* op. Bij patiënten met een langdurig verlaagde weerstand kan de *Aspergillus fumigatus* zich ontwikkelen tot een invasieve infectie waarbij weefsels en organen worden aangetast. Omdat *Aspergillus fumigatus* wordt ingeademd, zijn de longen het vaakst betrokken bij invasieve aspergillose. De infectie kan echter ook voorkomen in andere organen zoals de hersenen en de darmen.

Het vaststellen van de diagnose invasieve aspergillose kan moeilijk zijn en gebeurt vaak met behulp van de gereviseerde criteria van de European Organization for Research and Treatment of Cancer/Invasive Infectious Diseases Study Mycoses Group (EORTC/MSG). Hierbij wordt de het voorkomen van de schimmelziekte aangegeven met 'bewezen', 'waarschijnlijk' of 'mogelijk'. Bewezen invasieve aspergillose betekent dat er sprake is van een weefselbiopt met tekenen van een schimmelinfectie of dat *Aspergillus* groeit uit een kweek welke is afgenomen van steriel lichaamsmateriaal. Het is echter vaak niet mogelijk om een invasieve aspergillose te bewijzen vanwege de risico's op complicaties bij het afnemen van een biopt of omdat de patiënt te ziek is om een biopt te ondergaan. Daarom wordt de diagnose meestal indirect gesteld door middel van de criteria van 'waarschijnlijke' of 'mogelijke' invasieve aspergillose. Een 'waarschijnlijke' invasieve aspergillose bestaat uit drie verschillende criteria waaraan moet zijn voldaan: een vatbare patiënt, een klinisch criterium en een microbiologisch criterium. Een vatbare patiënt omvat een patiënt die een langdurig verlaagde weerstand heeft op basis van neutropenie (lage aantal van bepaald soort witte bloedcellen), immunosuppressiva (medicijnen die de weerstand verlagen) of een stamceltransplantatie. De klinische criteria omvatten o.a. specifieke afwijkingen in de longen, zoals de aanwezigheid van ronde afwijkingen met een halo- of crescent-teken. De microbiologisch criteria bestaan uit het aantonen van antistoffen tegen componenten van de *Aspergillus* celwand (galactomannan of Beta-(1,3)-d-glucan) of het kweken van *Aspergillus* uit niet steriel materiaal. Wanneer een patiënt voldoet aan één van elk van de patiënt, klinische en microbiologische criteria, dan is er sprake van een 'waarschijnlijke' invasieve aspergillose. Wanneer een patiënt alleen voldoet aan de patiënt en klinisch criteria, dan wordt er gesproken van een 'mogelijke' invasieve aspergillose. Zoals de benaming het aangeeft, hangt de zekerheid van de diagnose af van het aantal aanwezige criteria. De EORTC/

MSG criteria zijn opgesteld voor onderzoeksdoeleinden, maar worden in de kliniek ook gebruikt. Echter, zij omvatten niet alle patiënten die invasieve aspergillose hebben. Vatbare patiënten met een microbiologisch criterium, maar zonder een klinisch criterium, beschouwt men vaak toch als invasieve aspergillose hebbende. Deze patiënten worden ook zodanig behandeld, omdat onderzoek heeft aangetoond dat zij dezelfde complicaties en mortaliteit hebben als patiënten met een 'waarschijnlijke' invasieve aspergillose. Deze categorie bestaat niet in de EORTC/MSG criteria, maar wordt in dit proefschrift omschreven als 'non-classifiable' invasieve aspergillose.

Zoals eerder beschreven, hebben patiënten met een langdurig ernstig verlaagde weerstand een risico op het ontwikkelen van invasieve aspergillose. Eén van de vatbare patiëntengroepen zijn patiënten met een onderliggende hematologische ziekten (bloedziekten), die een langdurig verlaagde weerstand ontwikkelen door hun onderliggende ziekte (bijvoorbeeld acute leukemie) of door hun behandeling (intensieve chemotherapie of stamceltransplantatie). 2,3% tot 24% van de patiënten met acute leukemie of patiënten die een stamceltransplantatie hebben ondergaan, ontwikkelen een invasieve aspergillose. Een gedeelte van deze patiënten overlijdt aan de infectie. De eerstelijnsbehandeling bestaat uit voriconazol of posaconazol, welke medicijnen zijn uit de groep 'azolen'. Wanneer de diagnose invasieve aspergillose snel wordt gesteld en de behandeling met azolen direct wordt gestart, dan is de kans op overlijden relatief laag. Het is daarom belangrijk om de diagnose zo spoedig mogelijk te stellen, zodat adequate behandeling zonder vertraging gestart wordt.

In dit proefschrift is er gekeken naar de incidentie, mortaliteit en risicofactoren van invasieve aspergillose in patiënten met een onderliggende hematologische ziekte. Daarnaast werden nieuwe methoden om invasieve aspergillose te detecteren onderzocht.

INVASIEVE ASPERGILLOSE EN ALLOGENE STAMCELTRANSPLANTATIE

Een gedeelte van de patiënten met een onderliggende hematologische maligniteit wordt behandeld met een allogene stamceltransplantatie. Dit betekent dat er een transplantatie plaatsvindt met stamcellen van een geschikte donor. Deze donor kan een familielid of onbekende persoon zijn, of zelfs navelstrengbloed van pasgeborenen. Stamcellen bevinden zich normaliter in het beenmerg en ontwikkelen zich tot verschillende bloedcellen, zoals rode bloedcellen, witte bloedcellen en bloedplaatjes. Een allogene stamceltransplantatie zorgt niet alleen voor het herstel van de verschillende bloedcellen, maar helpt tevens in het behandelen van de onderliggende hematologische ziekte (Graft-versus-Disease effect). Patiënten die een allogene stamceltransplantatie ondergaan, hebben een langdurig verlaagde weerstand na de stamceltransplantatie. Enerzijds moet de weerstand opnieuw opgebouwd uit de donor stamcellen, anderzijds

is de weerstand verlaagd door medicijnen. Dit laatste is nodig omdat de witte bloedcellen van de donor ook tegen weefsels en organen van de patiënt kunnen keren, hetgeen omgekeerde afstotingsreactie oftewel Graft-versus-Host Disease wordt genoemd. Een medicijn dat veel wordt gebruikt voor de behandeling van Graft-versus-Host Disease, zijn corticosteroïden. Patiënten met een allogene stamceltransplantatie hebben een verhoogd risico op het ontwikkelen van invasieve aspergillose.

Het Erasmus Medisch Centrum heeft een grote afdeling hematologie waar allogene stamceltransplantaties plaatsvinden. In hoofdstuk 2 wordt een onderzoek beschreven waarin gekeken werd naar invasieve aspergillose bij patiënten die een allogene stamceltransplantatie hebben ondergaan in de periode van 2004 t/m 2014. Het doel van dit onderzoek was om naar de incidentie, mortaliteit en risicofactoren van invasieve aspergillose te kijken. In totaal kregen 663 patiënten een allogene stamceltransplantatie. Twaalf maanden na transplantatie hadden 86 van de 663 patiënten invasieve aspergillose ontwikkeld (1.8% bewezen, 7.4% waarschijnlijk en 3.8% non-classifiable invasieve aspergillose). De mortaliteit op 12 maanden was 14% hoger in patiënten met invasieve aspergillose in vergelijking met patiënten zonder invasieve aspergillose (40% versus 26%; p-waarde <0.01). Het bepalen van de risicofactoren werd gedaan door middel van een case-control onderzoek, waarbij de karakteristieken van patiënten met een invasieve aspergillose (case) vergeleken werden met patiënten zonder invasieve aspergillose (control). Er werden 99 case patiënten gepaard met 198 control patiënten op basis van leeftijd, jaar van stamceltransplantatie en duur van follow-up. De volgende risicofactoren werden gevonden: een verminderd aantal van neutrofiele granulocyten en lymfocyten (allebei zijn witte bloedcellen), een verminderde longfunctie voorafgaand aan de allogene stamceltransplantatie, een verminderde functie van de nieren, het reactiveren van cytomegalovirus en het gebruik van corticosteroïden bij de behandeling van Graft-versus-Host Disease. Een hogere dosis corticosteroïden werd geassocieerd met een hogere kans om invasieve aspergillose te ontwikkelen. Naar aanleiding van de resultaten uit dit onderzoek is het beleid aangepast betreffende de preventie van invasieve aspergillose. Patiënten die behandeld worden met een hoge dosis corticosteroïden krijgen nu ook therapie met azolen ter voorkoming van invasieve aspergillose.

INHALATIE VAN LIPOSOMAAL AMFOTERICINE-B TER VOORKOMING VAN INVASIEVE ASPERGILLOSE

Patiënten die een acute myeloïde leukemie hebben, worden behandeld met intensieve chemotherapie. Zij hebben enkele weken geen tot zeer lage aantallen van afweercellen na de chemotherapie. In deze periode zijn patiënten zeer vatbaar voor allerlei verschillende infectieziekten, waaronder invasieve aspergillose. In 2008 liet een onderzoek zien

dat het inhaleren van liposomaal amfotericine-B (L-AmB) de incidentie van invasieve aspergillose verlaagd van 14% naar 4%. L-AmB is een medicijn tegen invasieve aspergillose. In het onderzoek inhaleerden patiënten L-AmB 2x per week. Naar aanleiding van de bovenstaande resultaten wordt L-AmB inhalatie standaard voorgeschreven aan patiënten met acute myeloïde leukemie die intensieve chemotherapie ondergaan in het Erasmus Medisch Centrum. Hoofdstuk 3 beschrijft een prospectief onderzoek waarin we hebben gekeken naar de effectiviteit en kosten-baten van L-AmB inhalatie in patiënten met acute myeloïde leukemie, die behandeld werden met intensieve chemotherapie. In de periode van 2008 t/m 2012 waren er 127 patiënten die L-AmB hadden geïnhaleerd gedurende hun eerste en tweede chemotherapie. Deze 127 patiënten werden vergeleken met 108 historische controle patiënten die waren behandeld voor acute myeloïde leukemie in de periode van 2005 t/m 2008. De controle patiënten hadden geen L-AmB geïnhaleerd. Er werd een significante daling in de incidentie van bewezen en waarschijnlijke invasieve aspergillose gezien in de patiënten die L-AmB hadden geïnhaleerd (L-AmB groep 9,5% versus controle groep 34,4%; p-waarde=0,0064). Er werden geen ernstige bijwerkingen van de L-AmB inhalatie waargenomen. Daarnaast werden de kosten voor L-AmB en de bijbehorende apparatuur voor inhalatie ruimschoots gecompenseerd (1292 euro per patiënt) door de verminderde kosten in diagnostiek en gebruik van voriconazol (-1816 euro per patiënt). Concluderend was de inhalatie van L-AmB effectief ter voorkoming van invasieve aspergillose en daarbij ook kosteneffectief.

GALACTOMANNAN ANTIGEENTEST IN CEREBROSPINAAL VLOEISTOF

Invasieve aspergillose komt meestal voor in de longen, maar kan ook voorkomen in andere organen. Wanneer invasieve aspergillose in de hersenen voorkomt, wordt dit cerebrale aspergillose genoemd. Cerebrale aspergillose is zeldzaam en gaat gepaard met een zeer slechte overleving. De diagnose kan moeizaam zijn doordat het beeldvormend onderzoek van de hersenen niet specifiek is en *Aspergillus* in de hersenvloeistof slechts gekweekt wordt in minder dan een derde van de patiënten. Om de diagnose met zekerheid te stellen dient er een hersenbiopt afgenomen te worden. Dit is echter vaak niet mogelijk bij patiënten met een onderliggende hematologische ziekte vanwege potentiele complicaties of ernstig ziek zijn. Daarentegen is het vaak wel mogelijk om hersenvloeistof af te nemen middels een lumbaalpunctie. *Aspergillus* kan indirect worden aangetoond door de bepaling van antigenen tegen galactomannan, een celwand component welke vrijkomt tijdens de groei van *Aspergillus*. De galactomannan antigeentest behoort tot de microbiologische criteria van de EORTC/MSG criteria en kan op verschillende lichaamsmaterialen bepaald worden. De test is uitgebreid onderzocht op longvloeistof en bloed, maar er is weinig onderzoek verricht naar de bruikbaarheid

van deze test op hersenvloeistof. Hoofdstuk 4 beschrijft een onderzoek over de bruikbaarheid van de galactomannan antigeentest op hersenvloeistof. Alle patiënten van het Erasmus Medisch Centrum en Universitair Ziekenhuis Leuven werden geïncludeerd bij wie een galactomannan antigeentest op de hersenvloeistof was bepaald in de periode van 2004 t/m 2014. De EORTC/MSG criteria werden gebruikt om de diagnose bewezen of waarschijnlijke cerebrale aspergillose te stellen. Omdat de galactomannan antigeentest op hersenvloeistof werd onderzocht, werd deze geëxcludeerd van de microbiologische criteria. De galactomannan antigeentest was uitgevoerd op 205 hersenvloeistoffen van 157 patiënten. Bij een groot gedeelte van de patiënten waren de gegevens onvolledig. Deze patiënten werd daarom geëxcludeerd. Na exclusie bleven er 44 patiënten over, waarvan 4 patiënten met een bewezen cerebrale aspergillose, 13 patiënten met een waarschijnlijke cerebrale aspergillose en 27 patiënten zonder cerebrale aspergillose. Van de 17 patiënten met een cerebrale aspergillose hadden 15 patiënten een galactomannan antigeentest met een waarde van 2,0 of hoger. Van de patiënten zonder cerebrale aspergillose, hadden 26 van de 27 patiënten een galactomannan antigeentest van 0,5 of lager, en 1 patiënt een galactomannan antigeentest van 8,2. Wanneer een galactomannan cutoff waarde van 1,0 werd genomen, was de sensitiviteit 88,2% en de specificiteit 96,3%. We vonden dezelfde resultaten bij een cutoff waarde van 0,5 of 2,0. Concluderend heeft de galactomannan antigeentest een goede diagnostische waarde en kan de test gebruikt worden voor het aantonen of uitsluiten van cerebrale aspergillose.

AZOOL-RESISTENTE ASPERGILLUS FUMIGATUS

Azolen zijn de eerstelijnsbehandeling voor invasieve aspergillose. In de afgelopen 15 jaar is er sprake van een toenemende resistentie voor azolen. Deze azoolresistentie is ontstaan door overvloedig gebruik van azolen in de landbouw ter bescherming van de gewassen tegen schimmelinfecties. De agrarische azolen zijn qua chemische structuur vergelijkbaar met medische azolen, waardoor er kruisresistentie is ontstaan. Dit is een groot probleem, want onderzoek toont aan dat patiënten die geïnfecteerd zijn met een azoolresistente *Aspergillus fumigatus*, een zeer hoge mortaliteit van bijna 90% hebben. Azolen zijn bewezen superieur in de behandeling voor invasieve aspergillose ten opzichte van andere medicijnen als amfotericine-B en echinocandinen. Bovendien zijn azolen het enige anti-schimmel medicijn dat oraal gegeven kan worden, hetgeen van belang is omdat patiënten vaak langdurig behandeld moeten worden. Naast het probleem van therapie is de detectie van de azoolresistente *Aspergillus fumigatus* een uitdaging. Een gevoeligheidsbepaling naar anti-schimmel medicatie is mogelijk wanneer *Aspergillus* gekweekt is. *Aspergillus fumigatus* wordt echter in klein gedeelte van de patiënten gekweekt, waardoor een gevoeligheidsbepaling vaak niet mogelijk. Als

de *Aspergillus* wel wordt gekweekt, duurt het lang alvorens de gevoeligheidsbepaling bekend is. Dit komt omdat enerzijds de gevoeligheidsbepaling meestal gebeurt in een referentielaboratorium en anderzijds de *Aspergillus* langzaam groeit.

Het grootste gedeelte van de azoolresistentie wordt veroorzaakt door mutaties in het *Cyp51A* gen van de *Aspergillus fumigatus*. Het *Cyp51A* gen codeert voor het lanestrol 14-α-demethylase, een belangrijk enzym welke betrokken is in de bouw van de *Aspergillus* celwand. Dit enzym is tevens het aangrijpingspunt voor azolen. Mutaties in het *Cyp51A* gen kunnen zorgen voor azoolresistentie. Azoolresistentie in Nederland wordt voor bijna voor 90% veroorzaakt door twee mutatie combinaties in het *Cyp51A* gen: TR_{34}/L98H en TR_{46}/T298A/Y121F. Wanneer één van deze mutatie combinaties gevonden wordt, is er sprake van pan-azoolresistentie. Pan-azoolresistentie betekent dat er resistentie is tegen alle soorten azolen. Indien de twee mutatie combinaties snel aangetoond kunnen worden, is het mogelijk om een patiënt adequater te behandelen door het starten van een andere anti-schimmel therapie dan azolen.

Het aantonen van gen kan gebeuren door middel van een polymerase-kettingreactie (polymerase chain reaction: PCR). De PCR is een moleculaire techniek waarbij snel en eenvoudig specifieke stukken DNA vermenigvuldigd kunnen worden. Een multiplex-PCR is een PCR waarbij meerdere PCR producten tegelijkertijd aangemaakt worden. Idealiter zou een PCR voor het aantonen van *Aspergillus* DNA moeten voldoen aan twee voorwaarden: i) het aantonen van *Aspergillus* DNA direct op lichaamsmateriaal, ook wanneer er geen *Aspergillus* gekweekt wordt op datzelfde materiaal, en ii) het tevens aantonen van de meest voorkomende mutatie combinaties in het *Cyp51A* gen. Een dergelijke PCR is de AsperGenius® multiplex real-time PCR welke ontwikkeld werd door PathoNostics, een bedrijf in Maastricht. Deze commerciële PCR bestaat uit twee delen: de species PCR en de resistentie PCR. De species PCR toont de *Aspergillus fumigatus*, *Aspergillus terreus* en *Aspergillus* species aan. De resistentie PCR toont de mutaties TR_{34}, L98H, T298A en Y121F mutaties in het *Cyp51A* gen welke correleren met de twee meest voorkomende mutatie combinaties TR_{34}/L98H en TR_{46}/T298A/Y121F. In hoofdstuk 5 worden twee onderzoeken beschreven over de diagnostische waarde van de AsperGenius® PCR op broncho-alveolar lavage (BAL). Een BAL is vloeistof dat is verkregen na spoeling van de longen met water door middel van een bronchoscopie (longonderzoek). Dit longvloeistof wordt gebruikt voor een kweek en de bepaling van het galactomannan antigeentest. Hoofdstuk 5.1 beschrijft het eerste onderzoek waarbij de AsperGenius® PCR werd getest op 37 longvloeistoffen van patiënten met een onderliggende hematologische ziekte en 40 vloeistoffen van patiënten van de intensive care. Het onderzoek werd verricht in het Erasmus Medisch Centrum. De uitslagen van de AsperGenius® PCR werden vergeleken met de uitslagen van galactomannan antigeentesten en kweken welke eerder waren uitgevoerd op de longvloeistoffen. De positieve controles waren de longvloeistoffen met een galactomannan van ≥1,0 en/of een positieve *Aspergillus*

kweek. De negatieve controles waren de longvloeistoffen met een negatieve uitslag voor de galactomannan antigeentest en *Aspergillus* kweek. Tweeëntwintig van de 77 longvloeistoffen waren afkomstig van patiënten met invasieve aspergillose (2 bewezen, 9 waarschijnlijk en 11 non-classifiable). Negentien van de 22 longvloeistoffen waren positieve controles. Negen longvloeistoffen hadden een positieve *Aspergillus* kweek. Er werd gekeken naar de optimale cyclic threshold van de PCR. De cyclic threshold is het aantal vermenigvuldigingen die nodig zijn om een echt signaal te vinden dat *Aspergillus* aan te tonen. De optimale cyclic threshold waarde werd gevonden bij <36. Zestien van de 19 longvloeistoffen hadden een positieve species PCR, waarbij er 2 *Aspergillus* species en 14 *Aspergillus fumigatus* werden gevonden. Dit resulteerde in een sensitiviteit, specificiteit, positief en negatief voorspellende waarde van 88.9%, 89.3%, 72.7% en 96.2% in de hematologie groep en 80.0%, 93.3%, 80.0% en 93.3% in de intensive care groep. De resistentie PCR werd verricht op de 14 longvloeistoffen waar een *Aspergillus fumigatus* was gevonden. De resistentie PCR vond twaalf wildtypes (oftwel *Aspergillus fumigatus* zonder *Cyp51A* mutaties TR_{34}, L98H, T298A en Y121F welke meest waarschijnlijk gevoelig zijn voor azolen) en twee mutanten (één met TR_{34}/L98H en één met TR_{46}/Y121F/T289A). De twee longvloeistoffen met een *Aspergillus fumigatus* met mutaties waren afkomstig van patiënten die gefaald hadden op azooltherapie. Samenvattend had de geteste PCR een goede diagnostische waarde en was het mogelijk om de PCR te gebruiken op longvloeistoffen welke geen positieve *Aspergillus* kweek hadden. Daarbij kon de PCR *Cyp51A* mutaties in de *Aspergillus fumigatus* aantonen, welke gerelateerd zijn met azoolresistentie.

Het single-center onderzoek van hoofdstuk 5.1 is vervolgd door een tweede onderzoek. Hoofdstuk 5.2 beschrijft het vervolgonderzoek over de AsperGenius® PCR waarin vijf ziekenhuizen in Nederland en België deelnamen. In dit multicenter onderzoek waren de doelen: i) het bevestigen van de optimale cyclic threshold en diagnostische waarde van de PCR op longvloeistoffen, en ii) het onderzoeken of de aanwezigheid van resistentie geassocieerde mutaties (RAM) gerelateerd is met het falen van de behandeling met azolen of de mortaliteit op 6 weken na diagnose van invasieve aspergillose. Er waren 201 patiënten van wie een longvloeistof beschikbaar was. Achtentachtig van 201 longvloeistoffen waren positieve controles en 113 waren negatieve controles. De optimale cyclic threshold werd onderzocht middels twee verschillende methoden: de closest to (0,1) en de Youden index. Deze twee methoden gaven respectievelijk een cutoff van <38 en <36 aan als beste cyclic threshold. We kozen de cyclic threshold van <38 aan als optimale cutoff, omdat deze gepaard gaat met een hogere sensitiviteit. Het missen van de diagnose invasieve aspergillose heeft namelijk grote negatieve gevolgen voor een patiënt. Met een cyclic threshold cutoff van <38, hadden 74 van de 88 positieve controles een uitslag van de species PCR. Van deze 74 longvloeistoffen waren er bij 32 longvloeistoffen geen *Aspergillus* gekweekt. De sensitiviteit, specificiteit, positieve en

negatieve voorspellende waarden waren respectievelijk 84%, 80%, 76% and 87% bij een cyclic threshold cutoff van <38. Voor het bepalen van therapiefalen met azolen en mortaliteit op 6 weken werden de data van het eerste single-center onderzoek en het huidige onderzoek bij elkaar gevoegd. Dit werd gedaan omdat azoolresistentie nog relatief weinig voorkomt. In totaal waren er 78 patiënten die geïnfecteerd waren met wildtype *Aspergillus fumigatus* (n=65) of een *Aspergillus* fumigatus met RAMs (n=13). Vijventwintig van de 78 patiënten werden geëxcludeerd, omdat zij niet aan de vooraf opgestelde voorwaarden voldeden. Na de exclusie bleven er 53 patiënten over, waarvan 45 met een wildtype *Aspergillus fumigatus* en 8 met *Aspergillus fumigatus* met RAMs (6 TR_{34}/L98H en 2 met TR_{46}/Y121F/T289A). Het falen van azooltherapie werd gezien in 12 van de 45 patiënten met een wildtype *Aspergillus fumigatus* versus 6 van de 8 patiënten met *Aspergillus fumigatus* met RAMs (p-waarde = 0,01). De mortaliteit op 6 weken was 2,7 keer hoger in patiënten met een *Aspergillus fumigatus* met RAMs (18,6% zonder RAMs versus 50,0% met RAMs, p-waarde = 0,07). Hiermee werd voor het eerst beschreven dat er een associatie is tussen het falen van azooltherapie en het geïnfecteerd zijn met een *Aspergillus fumigatus* met RAMs.

ONDERSCHEIDING VAN *ASPERGILLUS FUMIGATUS, ASPERGILLUS LENTULUS* EN *ASPERGILLUS FELIS*

Er bestaan verschillende soorten *Aspergilli*. De *Aspergillus fumigatus* behoort tot de sectie *Fumigati* binnen de *Aspergilli*. De *Aspergilli* in de sectie *Fumigati* kunnen qua morfologie (uiterlijk) op elkaar lijken, waardoor onderscheiding alleen mogelijk is op basis van moleculaire techinieken. Het onderscheid tussen verschillende *Aspergilli* binnen de sectie *Fumigati* is belangrijk, omdat sommigen intrinsiek resistent zijn voor azolen. Intrinsieke resistentie betekent dat deze resistentie een stabiel genetisch eigenschap is en gecodeerd is in het DNA. Dit in tegenstelling tot verworven resistentie, welke verworven kan worden door bijvoorbeeld mutaties in het DNA. Tot op heden omvat de sectie *Fumigati* 63 species, waarvan er 17 beschreven zijn die invasieve aspergillose kunnen veroorzaken bij mensen en/of zoogdieren. Twee van deze beschreven *Aspergilli* zijn de *Aspergillus lentulus* en *Aspergillus felis.* Beiden zijn intrinsiek resistent voor azolen en worden sporadisch gerapporteerd als verwekkers van invasieve aspergillose.

In hoofdstuk 6 beschrijven wij twee patiënten die een bewezen invasieve aspergillose hadden ontwikkeld met respectievelijk een *Aspergillus lentulus* en *Aspergillus felis*. Deze twee *Aspergilli* waren gekweekt uit een biopt en identificatie werd verricht middels moleculaire technieken. De AsperGenius® PCR werd getest op deze twee *Aspergilli* en extra controle stammen van *Aspergillus fumigatus*, *Aspergillus lentulus* en *Aspergillus felis*. We onderzochten of de PCR deze drie *Aspergilli* kon onderscheiden, omdat *Aspergillus*

fumigatus meestal azoolgevoelig is en de *Aspergillus lentulus* en *Aspergillus felis* daarentegen juist azoolresistent zijn. Indien er geen onderscheid gemaakt kan worden, zou dit kunnen leiden tot een inadequate behandeling van een patiënt die geïnfecteerd is met een *Aspergillus lentulus* of *Aspergillus felis*.

De AsperGenius® resistentie PCR toonde variabele smeltpunten voor de drie verschillende *Aspergilli*, maar kon geen onderscheid maken. De PCR detecteerde geen TR_{34} marker in de *Aspergillus lentulus* en *Aspergillus felis*. Dit was in tegenstelling tot de *Aspergillus fumigatus* waarin de TR_{34} kon worden aangetoond zowel in een wildtype als mutant. Daarnaast waren de smeltpunten van de L98H en Y121F markers verschillend tussen *Aspergillus fumigatus*, *Aspergillus lentulus* en *Aspergillus felis*. Met name de Y121F marker was geschikt om de drie *Aspergilli* te onderscheiden. Dit resultaat werd ook geconfirmeerd door het testen van additionele isolaten (drie *Aspergillus fumigatus*, zes *Aspergillus lentulus* en twaalf *Aspergillus felis*). Concluderend was het mogelijk om *Aspergillus fumigatus*, *Aspergillus lentulus* en *Aspergillus felis* te onderscheiden middels de AsperGenius® PCR.

CONCLUSIE

In dit proefschrift zijn meerdere onderzoeken verricht naar invasieve aspergillose bij patiënten met een hematologische ziekte. Dit resulteerde in nieuwe inzichten in de epidemiologie, risicofactoren en diagnostiek van invasieve aspergillose. Risicofactoren voor het ontwikkelen van invasieve aspergillose in allogene stamceltransplantatie patiënten zijn neutropenie, lymfocytopenie, een verminderde functie van de nieren, een verminder longfunctie, het reactiveren van cytomegalovirus en het gebruikt van prednisolon. Het inhaleren van L-AmB voorkomt invasieve aspergillose in patiënten met acute myeloïde leukemie tijdens hun intensieve chemotherapie. Deze profylactische behandeling is tevens kosteneffectief. De galactomannan antigeentest heeft een goede diagnostische waarde in het cerebrospinal vloeistof en kan gebruikt worden voor het aantonen of uitsluiten van cerebrale aspergillose. De AsperGenius® PCR heeft een goede diagnostische waarde op longvloeistoffen, ook wanneer er geen *Aspergillus* groeit uit de longvloeistof. Daarbij kon de PCR *Cyp51A* mutaties aantonen welke correleren met azoolresistentie. Het aantonen van deze *Cyp51A* mutaties was tevens geassocieerd met het falen van azooltherapie. Ten slotte is het mogelijk om met deze PCR onderscheid te maken tussen *Aspergillus fumigatus*, *Aspergillus lentulus* en *Aspergillus felis*, drie verwante *Aspergilli* uit de sectie *Fumigati*.

Chapter 9

Additional information

LIST OF PUBLICATIONS

1 Chong GM, van de Sande WWJ, Dingemans GJH, Gaajetaan GR, Vonk AG, Hayette MP, et al. Validation of a New *Aspergillus* Real-Time PCR Assay for Direct Detection of *Aspergillus* and Azole Resistance of *Aspergillus fumigatus* on Bronchoalveolar Lavage Fluid. J Clin Microbiol. 2015;53(3):868-874.

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4 Chong GM, van der Beek MT, von dem Borne PA, Boelens J, Steel E, Kampinga GA, et al. PCR-based detection of Aspergillus fumigatus Cyp51A mutations on bronchoalveolar lavage: a multicenter validation of the AsperGenius assay in 201 patients with haematological disease suspected for invasive aspergillosis. J Antimicrob Chemother. 2016;71(12):3528-3535.

5 Chong GM, Vonk AG, Meis JF, Dingemans GJ, Houbraken J, Hagen F, et al. Interspecies discrimination of A. fumigatus and siblings A. lentulus and A. felis of the Aspergillus section Fumigati using the AsperGenius((R)) assay. Diagn Microbiol Infect Dis. 2017;87(3):247-252.

PHD PORTFOLIO

Courses

2014 Research integrity
2015 Good clinical practice
2015 Masterclass "Resistance and persistence in *Aspergillus fumigatus*"
2015 De 24-uur van Groenendael IFI
2016 De 24-uur van Groenendael IFI

Oral presentations at meetings and conferences

2014 Infectiologie symposium
2015 Masterclass "Resistance and persistence in *Aspergillus fumigatus*"
2015 Resistentie symposium
2016 Refereeravond LUMC
2016 6th European Congress of Clinical Microbiology and Infectious Diseases
2016 Themamiddag Werkgroep Moleculaire Diagnostiek en Infectieziekten

Poster presentations at conferences

2014 19th European Hematology Association congress
2015 7th Trends in Medical Mycology
2015 Wetenschapsdagen
2016 Wetenschapsdagen

Teaching experience

2015 Supervising master student

CURRICULUM VITAE

Ga-Lai Chong was born on the 21st of February 1982 in The Hague, the Netherlands. In 2000, she graduated from the Christelijk Gymnasium Sorghvliet in The Hague. Subsequently, she started medical school at the Erasmus University Rotterdam the same year. During medical school, she participated in the Research Master program of the Netherlands Institutes for Health Sciences. She investigated the association of HFE H63D mutation, Heberden's nodes and mortality in the Rotterdam Study under supervision of dr. B.Z. Alizadeh at the Department of Genetic Epidemiology, and completed her MSc in genetic epidemiology in 2004.

After receiving her medical degree in November 2006, she started as a resident in Internal Medicine under supervision of dr. M.A. van den Dorpel at the Maasstad Ziekenhuis, Rotterdam. Subsequently in May 2011, she continued her residency in Internal Medicine and Hematology at the Erasmus University Medical Center, Rotterdam (prof. dr. J.L.C.M. van Saase, dr. S.C.E. Klein Nagelvoort-Schuit, prof. dr. J.J. Cornelissen and dr. P.A.W. te Boekhorst). Ga-Lai interrupted her residency in January 2013 and in September 2014 to work on this thesis at the Department of Internal Medicine, section of Infectious Diseases, Erasmus University Medical Center. Her research focused on the incidence, mortality, prevention and diagnostics of invasive aspergillosis in patients with an underlying hematological disease. She was supervised by dr. B.J.A. Rijnders in collaboration with prof. dr. A. Verbon and prof. dr. J.J. Cornelissen.

In November 2015, Ga-Lai finished her training as internist-hematologist and worked in the Admiraal de Ruyter Ziekenhuis in Goes until March 2016. After finishing her research in August 2016, she started as a resident in Medical Microbiology in January 2017, which takes part in the Erasmus University Medical Center (dr. A.G. Vonk) and Reinier de Graaf Gasthuis, Delft (dr. R. Vreede and dr. L.C. Smeets).

DANKWOORD

Een proefschrift komt tot stand door de inzet en wilskracht van veel mensen. Allereerst dank ik de patiënten voor hun deelname aan de verschillende onderzoeken. Daarnaast wil ik nog een aantal mensen in het bijzonder bedanken.

Graag wil ik mijn co-promotor Bart Rijnders bedanken. Beste Bart, toen ik mijn consultenstage infectieziekten kwam doen op de afdeling, had ik niet gedacht dat ik uiteindelijk zou blijven om te promoveren. Je daadkracht en snelheid hebben mij vaak versteld laten staan. Vele dank dat ik bij jou onderzoek mocht doen. Ik heb veel van je geleerd.

Beste prof. dr. Verbon, beste Annelies, je bewaakte de tijdslijn tijdens mijn promotie traject. Toen ik langs kwam om aan te geven dat ik een carrière switch wilde maken, luisterde je rustig naar mijn verhaal en gaf mij goed advies. Hartelijk dank dat jij mijn promotor wilt zijn.

Beste prof. dr. Cornelissen, beste Jan, ik heb mijn opleiding tot internist-hematoloog onder jouw begeleiding afgerond. Tijdens de opleiding gaf je mij de gelegenheid om tijdelijk de opleiding te onderbreken om aan dit onderzoek te werken. Ik dank je dat je dit mogelijk hebt gemaakt en dat je mijn promotor wilt zijn.

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

Vele dank aan alle coauteurs voor jullie bijdragen aan de verschillende studies en het becommentariëren van de manuscripten. Daarnaast ben ik voor verschillende studies in andere studiecentra in Nederland en België geweest. Altijd werd ik hartelijk ontvangen door de medeonderzoekers en ondersteunende personeel en werd ik geholpen bij het terugzoeken van gegevens en BAL-vloeistoffen. Bedankt voor jullie vriendelijke hulp waardoor ik in korte tijd alles bij elkaar kon zoeken. Ook bedank ik Fleur Broekman, René van Engen en Jaap Kuipers: in tijden dat ik weinig tijd had om gegevens op te zoeken, hebben jullie mij geholpen met het opzoeken van data. Vele dank voor jullie hulp!

Ook dank aan de medewerkers van Pathonostics voor het verwerken van alle BAL vloeistoffen in de twee studies. Met name Gijs en Giel wil ik extra noemen: met jullie tweeën heb ik het meest gediscussieerd over de AsperGenius PCR. Bedankt voor jullie snelle en prettige samenwerking.

Tevens dank aan de collega's van de hematologie: hartelijk dank aan mijn supervisoren, ik heb veel van jullie geleerd op de centrum locatie en Daniel den Hoed Kliniek. Aan de (oud) arts-assistenten, bij wijlen en tijden was de hematologie stage zwaar. Ik ben blij dat we veel steun aan elkaar hadden. Hartelijk dank voor jullie gezelligheid en aanwezigheid. Drie collegae wil ik nog extra noemen: Gerard, al vanaf het Maasstad ziekenhuis kennen wij elkaar. Dank je voor je gezelligheid en goede adviezen. Heel veel succes met je klinische en wetenschappelijke carrière. Jurjen, hartelijk dank voor je analyses en commentaren, ons stuk is er een stuk beter op geworden. Veel succes met het afronden van je opleiding. Tim, bedankt voor je gezelligheid. Ik wens je heel veel succes met het afronden van je proefschrift, maar dat moet zeker lukken met jouw prachtige publicatie in de New England Journal of Medicine.

Vele dank aan alle collega's van de MMIZ. Ik ben blij dat ik mijn opleiding tot arts-microbioloog in het Erasmus MC doe. De sfeer is laagdrempelig en fijn. In het bijzonder wil ik nog de arts-assistenten en Alieke noemen: aan de arts-assistenten, toen ik tijdens mijn promotieonderzoek uit de Z-flat moest, werd ik gelukkig verwelkomd bij jullie op de kamer. Dank jullie allen voor de gezelligheid. Aan Alieke, toen ik langs kwam om te praten vanwege een carrière switch van hematologie naar medische microbiologie, zei je dat ik vooral mijn gevoel moest volgen. Ik ben blij dat ik je advies heb opgevolgd en heb geen spijt van mijn switch. Ik dank je hartelijk en ben blij dat jij mijn opleidster bent.

Beste mede-promovendi, beste Ingeborg, Anne, Hassna, Sabrina, Alexander, Bas en Casper, tijdens mijn promotieonderzoek ben ik menigmaal verhuisd tussen de Z-flat, de D-vleugel en het Na-gebouw. Maar waar ik ook zat, ik kon altijd rekenen op jullie wanneer ik vragen had over onderzoeks-gerelateerde zaken. Ik dank jullie allen en succes aan diegenen die hun onderzoek nog moeten afronden.

Beste vrienden, altijd was het goed als ik even wilde praten over mijn promotieonderzoek en welke hindernis er dan weer op het pad lag. Dank jullie allen voor jullie aanwezigheid en gezelligheid.

Dear family from Hong Kong, when we are visiting Hong Kong, you always warmly welcome us and take us to different places. Thank you all for your kind hospitality.

Lieve Anoesh, ondanks dat de afgelopen jaren moeilijk waren, is onze vriendschap gebleven. In de loop der jaren steunde jij mij bij verschillende belangrijke gebeurtenissen. Ik dank je dat jij mijn paranimf bent en wederom naast mij wilt staan om mij te steunen. Ik wens jou, Kyran en Michael veel geluk, op welke manier jullie ook samen zijn.

Lieve Marlies en Ynze, lieve papa en mama, ik ben blij dat ik zulke lieve schoonouders heb die altijd geïnteresseerd zijn in hetgeen wat me bezighoudt en voor Boudewijn en mij klaar staan. Beste Caroline, Bart en Domino, het is altijd gezellig om bij jullie te zijn en superlekkere kip uit de oven te mogen eten. Lieve Kiek, onder uw handen wordt de tuin mooier en komen er steeds meer vogels aanvliegen. U heeft altijd een luisterend oor en geeft goed advies. U bent de oma die ik nooit heb gehad als kind. Ik hoop dat we nog veel Monopolie mogen spelen (en vaak verbonden kunnen sluiten om Boudewijn te verslaan). Wat een geluk dat ik zo'n lieve schoonfamilie heb.

Lieve Fung, lieve broer, ondanks dat je ooit hebt gezegd dat je nooit meer ceremoniemeester of iets in die aard wilde zijn, ben ik blij dat je toch hebt toegestemd om mijn paranimf te zijn. Je aanwezigheid met goede cynische grappen is belangrijk voor mij. Dankjewel voor je steun. Ik wens je veel succes met alle werkzaamheden. Aan mamma: 媽媽，我相信你看見女兒的成就，感到非常驕傲。謝謝你多年的愛護及耐心。若是，爸爸今天能看見一切，相信他也會為他的一雙兒女以感到自豪。

Lieve Boudewijn, je bent mijn steun en toeverlaat. Ik ben gezegend dat we samen zijn. Ik hoop dat we samen nog vele wandelingen hand in hand mogen maken in de wijde natuur.