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-

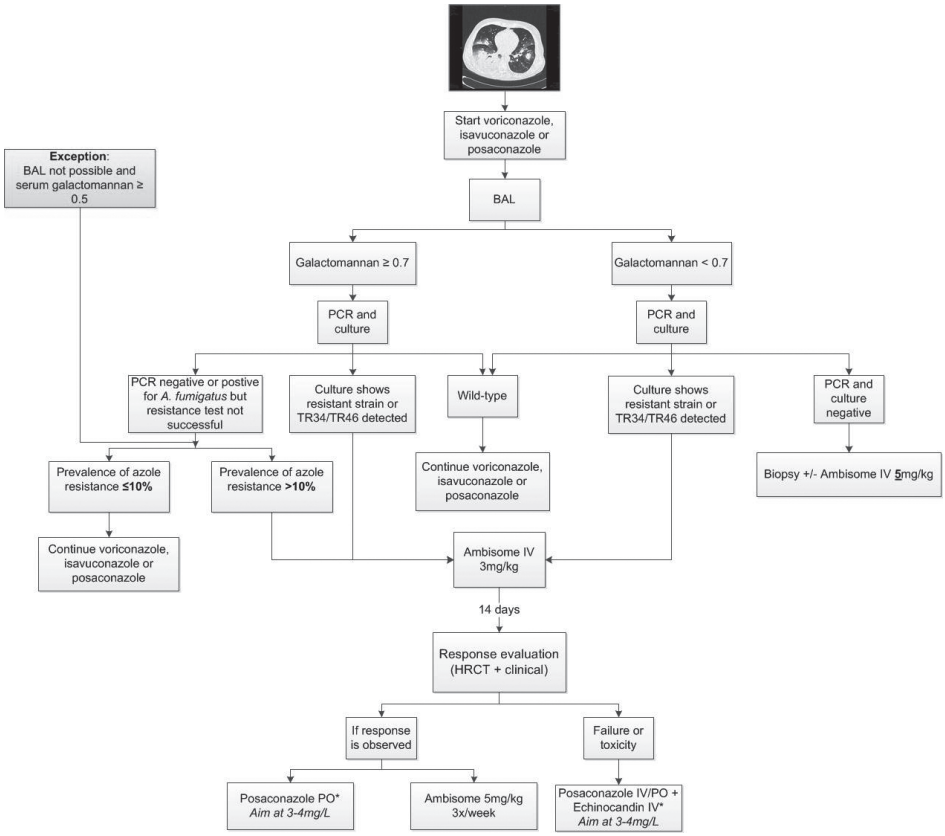

Figure 1. Diagnostic and therapeutic protocol used in the AzoRMan study.
A minimum inhibitory concentration of >2 in culture susceptibility testing means that the *A. fumigatus* is voriconazole resistant according to the method of the Clinical Laboratory Standards Institute. TR_{34}/TR_{46} means that 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.

REFERENCES

1 Kontoyiannis DP, Marr KA, Park BJ, Alexander BD, Anaissie EJ, Walsh TJ, et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clin Infect Dis. 2010;50(8):1091-1100.

2 Pagano L, Caira M, Candoni A, Offidani M, Fianchi L, Martino B, et al. The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. Haematologica. 2006;91(8):1068-1075.

3 Denning DW. Therapeutic outcome in invasive aspergillosis. Clin Infect Dis. 1996;23(3):608-615.

4 Lin SJ, Schranz J, Teutsch SM. Aspergillosis case-fatality rate: systematic review of the literature. Clin Infect Dis. 2001;32(3):358-366.

5 Slobbe L, Polinder S, Doorduijn JK, Lugtenburg PJ, el Barzouhi A, Steyerberg EW, et al. Outcome and medical costs of patients with invasive aspergillosis and acute myelogenous leukemia-myelodysplastic syndrome treated with intensive chemotherapy: an observational study. Clin Infect Dis. 2008;47(12):1507-1512.

6 Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann JW, et al. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med. 2002;347(6):408-415.

7 Patterson TF, Thompson GR, 3rd, Denning DW, Fishman JA, Hadley S, Herbrecht R, et al. Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America. Clin Infect Dis. 2016;63(4):e1-e60.

8 Warris A, Weemaes CM, Verweij PE. Multidrug resistance in *Aspergillus fumigatus*. N Engl J Med. 2002;347(26):2173-2174.

9 de Greeff SC, Mouton JW. NethMap 2017: Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands / MARAN 2017: Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2016. Nethmap / Maran 2017: Rijksinstituut voor Volksgezondheid en Milieu RIVM.

10 Vermeulen E, Lagrou K, Verweij PE. Azole resistance in *Aspergillus fumigatus*: a growing public health concern. Curr Opin Infect Dis. 2013;26(6):493-500.

11 Verweij PE, Chowdhary A, Melchers WJ, Meis JF. Azole resistance in *Aspergillus fumigatus*: can we retain the clinical use of mold-active antifungal azoles? Clin Infect Dis. 2016;62(3):362-368.

12 Corzo-Leon DE, Satlin MJ, Soave R, Shore TB, Schuetz AN, Jacobs SE, et al. Epidemiology and outcomes of invasive fungal infections in allogeneic haematopoietic stem cell transplant recipients in the era of antifungal prophylaxis: a single-centre study with focus on emerging pathogens. Mycoses. 2015;58(6):325-336.

13 Garcia-Vidal C, Upton A, Kirby KA, Marr KA. Epidemiology of invasive mold infections in allogeneic stem cell transplant recipients: biological risk factors for infection according to time after transplantation. Clin Infect Dis. 2008;47(8):1041-1050.

14 Girmenia C, Raiola AM, Piciocchi A, Algarotti A, Stanzani M, Cudillo L, et al. Incidence and outcome of invasive fungal diseases after allogeneic stem cell transplantation: a prospective study of the Gruppo Italiano Trapianto Midollo Osseo (GITMO). Biol Blood Marrow Transplant. 2014;20(6):872-880.

15 Harrison N, Mitterbauer M, Tobudic S, Kalhs P, Rabitsch W, Greinix H, et al. Incidence and characteristics of invasive fungal diseases in allogeneic hematopoietic stem cell transplant recipients: a retrospective cohort study. BMC Infect Dis. 2015;15(584.

16 Mikulska M, Raiola AM, Bruno B, Furfaro E, Van Lint MT, Bregante S, et al. Risk factors for invasive aspergillosis and related mortality in recipients of allogeneic SCT from alternative donors: an analysis of 306 patients. Bone Marrow Transplant. 2009;44(6):361-370.
17 Neofytos D, Treadway S, Ostrander D, Alonso CD, Dierberg KL, Nussenblatt V, et al. Epidemiology, outcomes, and mortality predictors of invasive mold infections among transplant recipients: a 10-year, single-center experience. Transpl Infect Dis. 2013;15(3):233-242.
18 Nucci M, Garnica M, Gloria AB, Lehugeur DS, Dias VC, Palma LC, et al. Invasive fungal diseases in haematopoietic cell transplant recipients and in patients with acute myeloid leukaemia or myelodysplasia in Brazil. Clin Microbiol Infect. 2013;19(8):745-751.
19 Omer AK, Ziakas PD, Anagnostou T, Coughlin E, Kourkoumpetis T, McAfee SL, et al. Risk factors for invasive fungal disease after allogeneic hematopoietic stem cell transplantation: a single center experience. Biol Blood Marrow Transplant. 2013;19(8):1190-1196.
20 Pagano L, Caira M, Nosari A, Van Lint MT, Candoni A, Offidani M, et al. Fungal infections in recipients of hematopoietic stem cell transplants: results of the SEIFEM B-2004 study--Sorveglianza Epidemiologica Infezioni Fungine Nelle Emopatie Maligne. Clin Infect Dis. 2007;45(9):1161-1170.
21 Neofytos D, Horn D, Anaissie E, Steinbach W, Olyaei A, Fishman J, et al. Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of Multicenter Prospective Antifungal Therapy (PATH) Alliance registry. Clin Infect Dis. 2009;48(3):265-273.
22 Liu YC, Chien SH, Fan NW, Hu MH, Gau JP, Liu CJ, et al. Incidence and risk factors of probable and proven invasive fungal infection in adult patients receiving allogeneic hematopoietic stem cell transplantation. J Microbiol Immunol Infect. 2015.
23 Gergis U, Markey K, Greene J, Kharfan-Dabaja M, Field T, Wetzstein G, et al. Voriconazole provides effective prophylaxis for invasive fungal infection in patients receiving glucocorticoid therapy for GVHD. Bone Marrow Transplant. 2010;45(4):662-667.
24 Ullmann AJ, Lipton JH, Vesole DH, Chandrasekar P, Langston A, Tarantolo SR, et al. Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. N Engl J Med. 2007;356(4):335-347.
25 Sun Y, Meng F, Han M, Zhang X, Yu L, Huang H, et al. Epidemiology, management, and outcome of invasive fungal disease in patients undergoing hematopoietic stem cell transplantation in China: a multicenter prospective observational study. Biol Blood Marrow Transplant. 2015;21(6):1117-1126.
26 Zhang P, Jiang EL, Yang DL, Yan ZS, Huang Y, Wei JL, et al. Risk factors and prognosis of invasive fungal infections in allogeneic stem cell transplantation recipients: a single-institution experience. Transpl Infect Dis. 2010;12(4):316-321.
27 De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis. 2008;46(12):1813-1821.
28 Haiduven D. Nosocomial aspergillosis and building construction. Med Mycol. 2009;47 Suppl 1(S210-216.
29 Oren I, Haddad N, Finkelstein R, Rowe JM. Invasive pulmonary aspergillosis in neutropenic patients during hospital construction: before and after chemoprophylaxis and institution of HEPA filters. Am J Hematol. 2001;66(4):257-262.

30 Rijnders BJ, Cornelissen JJ, Slobbe L, Becker MJ, Doorduijn JK, Hop WC, et al. Aerosolized liposomal amphotericin B for the prevention of invasive pulmonary aspergillosis during prolonged neutropenia: a randomized, placebo-controlled trial. Clin Infect Dis. 2008;46(9):1401-1408.
31 Maertens J, Marchetti O, Herbrecht R, Cornely OA, Fluckiger U, Frere P, et al. European guidelines for antifungal management in leukemia and hematopoietic stem cell transplant recipients: summary of the ECIL 3--2009 update. Bone Marrow Transplant. 2011;46(5):709-718.
32 Schwartz S, Behre G, Heinemann V, Wandt H, Schilling E, Arning M, et al. Aerosolized amphotericin B inhalations as prophylaxis of invasive aspergillus infections during prolonged neutropenia: results of a prospective randomized multicenter trial. Blood. 1999;93(11):3654-3661.
33 Griese M, Schams A, Lohmeier KP. Amphotericin B and pulmonary surfactant. Eur J Med Res. 1998;3(8):383-386.
34 Stichting Werkgroep Antibioticabeleid. SWAB Guidelines for the Management of Invasive Fungal Infection 2017 [cited 2018 10 Jan]. Available from: http://www.swab.nl/richtlijnen.
35 Jantunen E, Volin L, Salonen O, Piilonen A, Parkkali T, Anttila VJ, et al. Central nervous system aspergillosis in allogeneic stem cell transplant recipients. Bone Marrow Transplant. 2003;31(3):191-196.
36 Antinori S, Corbellino M, Meroni L, Resta F, Sollima S, Tonolini M, et al. *Aspergillus* meningitis: a rare clinical manifestation of central nervous system aspergillosis. Case report and review of 92 cases. J Infect. 2013;66(3):218-238.
37 Kourkoumpetis TK, Desalermos A, Muhammed M, Mylonakis E. Central nervous system aspergillosis: a series of 14 cases from a general hospital and review of 123 cases from the literature. Medicine (Baltimore). 2012;91(6):328-336.
38 Kami M, Ogawa S, Kanda Y, Tanaka Y, Machida U, Matsumura T, et al. Early diagnosis of central nervous system aspergillosis using polymerase chain reaction, latex agglutination test, and enzyme-linked immunosorbent assay. Br J Haematol. 1999;106(2):536-537.
39 Viscoli C, Machetti M, Gazzola P, De Maria A, Paola D, Van Lint MT, et al. *Aspergillus* galactomannan antigen in the cerebrospinal fluid of bone marrow transplant recipients with probable cerebral aspergillosis. J Clin Microbiol. 2002;40(4):1496-1499.
40 Steinmann J, Hamprecht A, Vehreschild MJ, Cornely OA, Buchheidt D, Spiess B, et al. Emergence of azole-resistant invasive aspergillosis in HSCT recipients in Germany. J Antimicrob Chemother. 2015;70(5):1522-1526.
41 van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, et al. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007-2009. Emerg Infect Dis. 2011;17(10):1846-1854.
42 Marr KA, Schlamm HT, Herbrecht R, Rottinghaus ST, Bow EJ, Cornely OA, et al. Combination antifungal therapy for invasive aspergillosis: a randomized trial. Ann Intern Med. 2015;162(2):81-89.
43 Meersseman W, Lagrou K, Maertens J, Wilmer A, Hermans G, Vanderschueren S, et al. Galactomannan in bronchoalveolar lavage fluid: a tool for diagnosing aspergillosis in intensive care unit patients. Am J Respir Crit Care Med. 2008;177(1):27-34.
44 Bader O, Weig M, Reichard U, Lugert R, Kuhns M, Christner M, et al. cyp51A-Based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. Antimicrob Agents Chemother. 2013;57(8):3513-3517.
45 Fuhren J, Voskuil WS, Boel CH, Haas PJ, Hagen F, Meis JF, et al. High prevalence of azole resistance in *Aspergillus fumigatus* isolates from high-risk patients. J Antimicrob Chemother. 2015;70(2894-2898.

46 Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, et al. Frequency and evolution of Azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerg Infect Dis. 2009;15(7):1068-1076.

47 Lockhart SR, Frade JP, Etienne KA, Pfaller MA, Diekema DJ, Balajee SA. Azole resistance in *Aspergillus fumigatus* isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the cyp51A gene. Antimicrob Agents Chemother. 2011;55(9):4465-4468.

48 Ozmerdiven GE, Ak S, Ener B, Agca H, Cilo BD, Tunca B, et al. First determination of azole resistance in *Aspergillus fumigatus* strains carrying the TR34/L98H mutations in Turkey. J Infect Chemother. 2015;21(8):581-586.

49 van der Linden JW, Arendrup MC, Warris A, Lagrou K, Pelloux H, Hauser PM, et al. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. Emerg Infect Dis. 2015;21(6):1041-1044.

50 Chowdhary A, Sharma C, Hagen F, Meis JF. Exploring azole antifungal drug resistance in *Aspergillus fumigatus* with special reference to resistance mechanisms. Future Microbiol. 2014;9(5):697-711.

51 Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. Eukaryot Cell. 2005;4(3):625-632.

52 Coelho D, Silva S, Vale-Silva L, Gomes H, Pinto E, Sarmento A, et al. *Aspergillus viridinutans*: an agent of adult chronic invasive aspergillosis. Med Mycol. 2011;49(7):755-759.

53 Pelaez T, Alvarez-Perez S, Mellado E, Serrano D, Valerio M, Blanco JL, et al. Invasive aspergillosis caused by cryptic *Aspergillus* species: a report of two consecutive episodes in a patient with leukaemia. J Med Microbiol. 2013;62(Pt 3):474-478.

54 Frisvad JC, Larsen TO. Extrolites of *Aspergillus fumigatus* and Other Pathogenic Species in *Aspergillus* Section *Fumigati*. Front Microbiol. 2015;6(1485.

55 Alhambra A, Catalan M, Moragues MD, Brena S, Ponton J, Montejo JC, et al. Isolation of *Aspergillus lentulus* in Spain from a critically ill patient with chronic obstructive pulmonary disease. Rev Iberoam Micol. 2008;25(4):246-249.

56 Escribano P, Pelaez T, Munoz P, Bouza E, Guinea J. Is azole resistance in *Aspergillus fumigatus* a problem in Spain? Antimicrob Agents Chemother. 2013;57(6):2815-2820.

57 Montenegro G, Sanchez Puch S, Jewtuchowicz VM, Pinoni MV, Relloso S, Temporitti E, et al. Phenotypic and genotypic characterization of *Aspergillus lentulus* and *Aspergillus fumigatus* isolates in a patient with probable invasive aspergillosis. J Med Microbiol. 2009;58(Pt 3):391-395.

58 Zbinden A, Imhof A, Wilhelm MJ, Ruschitzka F, Wild P, Bloemberg GV, et al. Fatal outcome after heart transplantation caused by *Aspergillus lentulus*. Transpl Infect Dis. 2012;14(5):E60-63.

59 Verweij PE, Ananda-Rajah M, Andes D, Arendrup MC, Bruggemann RJ, Chowdhary A, et al. International expert opinion on the management of infection caused by azole-resistant Aspergillus fumigatus. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy. 2015;21-22(30-40.

60 Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? Lancet Infect Dis. 2009;9(12):789-795.

61 U.S. National Library of Medicine. PCR Based Detection of Azole Resistance in A. Fumigatus to Improve Patient Outcome. (AzorMan) 2017 [cited 2018 10 Jan]. Available from: https://clinicaltrials.gov/ct2/show/NCT03121235.

62 White PL, Posso RB, Barnes RA. Analytical and Clinical Evaluation of the PathoNostics AsperGenius Assay for Detection of Invasive Aspergillosis and Resistance to Azole Antifungal Drugs during Testing of Serum Samples. J Clin Microbiol. 2015;53(7):2115-2121.

63 White PL, Posso RB, Barnes RA. Analytical and Clinical Evaluation of the PathoNostics AsperGenius Assay for Detection of Invasive Aspergillosis and Resistance to Azole Antifungal Drugs Directly from Plasma Samples. J Clin Microbiol. 2017;55(8):2356-2366.