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

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*No author list is provided because this is an unpublished interim analysis of an ongoing study*

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

## ABSTRACT


### Introduction

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

### Methods

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

### Results

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

## Conclusion

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

## INTRODUCTION

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

## METHODS

### Study design

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

### AsperGenius® PCR

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

### Treatment protocol (figure 1)

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

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

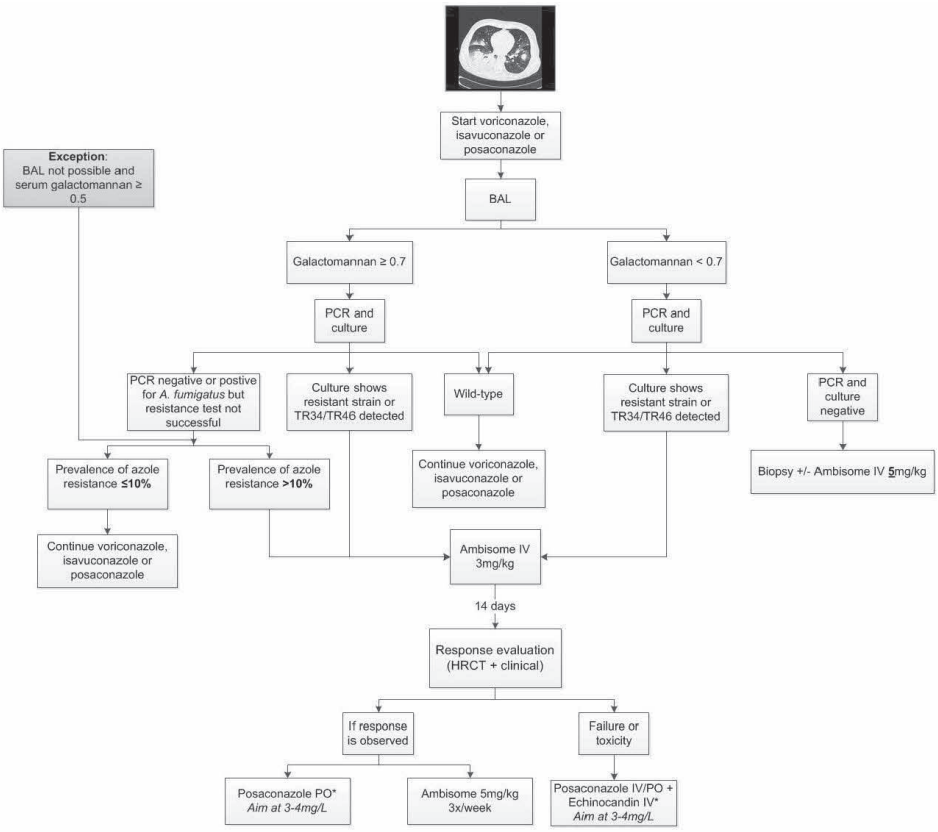


**Figure 1:** Treatment protocol for Azole Resistance Management (AzoRMan)-study.
MIC=Minimal Inhibitory concentration; IV=Intravenously. *Posaconazole HD can only be considered as treatment option when the MIC (EUCAST) ≤ 1g/dL. HRCT= High Resolution CT scan, PCR=polymerase chain reaction, PO=by mouth,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

## Primary and secondary endpoints

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

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

### Sample size calculation

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

## RESULTS

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

### Baseline characteristics

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

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

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

## Performance diagnostics

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

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

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

## Outcome

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

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

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

## DISCUSSION

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

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

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

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

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

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

## CONCLUSION

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

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

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