The prevalence of invasive aspergillosis (IA) at the intensive care unit (ICU) is unknown and difficult to assess since IA also develops in patients lacking specific host factors. In the Netherlands, increasing azole-resistance in Aspergillus fumigatus complicates treatment of patients with IA. The aim of this study was to determine the prevalence of IA by azole-resistant A. fumigatus at the ICU among patients receiving antifungal treatment and to follow their clinical outcome and prognosis. A retrospective cohort study was conducted in a university hospital ICU from January 2010 to December 2013. From all patients who received antifungal treatment for suspected IA, relevant clinical and microbiological data were collected using a standardised questionnaire. Of 9,121 admitted ICU-patients, 136 had received antifungal treatment for suspected IA, of which 38 had a positive A. fumigatus culture. Ten of the 38 patients harboured at least one azole-resistant isolate. Resistance mechanisms consisted of alterations in the cyp51A gene, more specific TR34/L98H and TR46/Y121F/T289A. Microsatellite typing did not show clonal relatedness, though isolates from two patients were genetically related. The overall 90-day mortality of patients with IA by azole-resistant A. fumigatus and patients with suspicion of IA by azole-susceptible isolates in the ICU was 100% (10/10) vs 82% (23/28) respectively. We conclude that the changing pattern of IA in ICU patients requires appropriate criteria for recognition, diagnosis and rapid resistance tests. The increase in azole resistance rates also challenges a reconsideration of empirical antifungal therapy.

Introduction
Azole resistance is an emerging problem in Aspergillus infections caused by Aspergillus fumigatus, with increasing reports of azole treatment failure [1-6]. Surveillance studies increasingly report geographical spread of azole resistance in environmental and clinical A. fumigatus isolates, including in Europe, Asia, the Middle East, Africa and most recently North and South America [4,8-10]. In contrast, surveillance studies from the Netherlands revealed azole resistance to be endemic, with dominance of the TR34/L98H and TR46/Y121F/T289A cyp51A-gene mediated resistance mechanisms [3]. These studies were based on routinely screening for the presence of azole resistance in all Aspergillus spp. isolates cultured from clinical samples in five university hospitals, irrespective of the clinical relevance of the culture result [3]. Although these surveys provide insight in the overall prevalence of azole resistance in culture-positive patients, the implications of azole resistance for specific patient groups remain unknown. In recent years, new categories of patients, lacking specific host factors as defined by the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG), such as intensive care unit (ICU) patients, are increasingly recognised for their susceptibility to develop IA. Thus the reported prevalence of invasive aspergillosis (IA) has increased at the ICU [11-13]. As information on IA by azole-resistant A. fumigatus in the ICU is scarce, we performed a retrospective cohort study in order to investigate the prevalence of azole resistance and its implications for patient treatment.

Methods
Study population
A retrospective cohort study was conducted in a 33-bed tertiary university hospital ICU in the Netherlands. Patients were identified by running a query for the period from January 2010 to December 2013 as those prescribed with voriconazole, conventional amphotericin B (c-AmB) or liposomal amphotericin B (L-AmB). Patients receiving mold-active drugs as prophylaxis only or antifungal therapy for non-Aspergillus fungal
infections were excluded. Furthermore, patients suspected for IA, but where *A. fumigatus* could not be grown, were excluded as well. Thus, the study population consisted of ICU patients who were treated for suspected IA and in whom *A. fumigatus* was cultured, since resistance testing can only be done on cultured isolates.

**Ethical consideration**
Due to the retrospective design and report on anonymous patient data, ethical approval was not required.

**Diagnosis of invasive aspergillosis**
All patients with a clinical syndrome suspected for IA underwent computed tomography (CT)-scanning and bronchoscopy for histological and microbiological examinations, unless clinical respiratory condition did not allow interventions or transportation. In addition to the bronchoalveolar lavage (BAL) or lung biopsy, supplementary samples were occasionally available for galactomannan test or culture, such as serum, tracheal aspirate or sputum. Antifungal treatment was administered to all patients with specific CT lesions (among others halo sign) and a positive culture or *Aspergillus* biomarker in serum or BAL. Since CT lesions are only present in a minority of the ICU population [14], patients with a high clinical suspicion of IA (based on host characteristics and exclusion of an alternative diagnosis) and a positive respiratory or serum sample were treated as well. Following local and national guidelines, first choice treatment was voriconazole. When azole resistance was suspected or severe abnormal liver function tests were present, L-AmB or c-AmB were prescribed.

**Data collection**
Electronic patient records (EZIS-Chipsoft, iMDsoft, GLIMS) were searched by the investigators (AitV, AR, JvP). Data regarding host factors, CT reports, microbiology results, mortality and use of prior antifungal therapy in the past 6 months were extracted from the hospital information system using a pre-assessed questionnaire. The EORTC/MSG criteria were applied to categorise patients as proven IA, probable IA or unclassified [15]. Since these criteria have been reported as less suitable for ICU patients, the ICU-criteria of Blot et al. were also used to classify patients with proven IA, putative IA or colonisation [16].

**Microbiology**
BAL fluids were tested for galactomannan (Platelia Aspergillus EIA, Bio-Rad, the Netherlands) using the recommended cut-off index of 0.5 for positivity. Direct microscopy was performed using the optical brightener calcofluor white on the sediment of the BAL fluid. Cultures for yeast and fungi were performed using standard techniques [17], including use of a selective Sabouraud dextrose agar incubated for 1 week at 35°C. If the culture was positive, *Aspergillus* species were identified to the species complex level using macroscopic and microscopic morphology. In addition, the ability of the fungus to grow at 48°C was used to identify *A. fumigatus* species complex.

From cultures yielding *A. fumigatus*, up to four colonies were subcultured on a four-well agar plate to screen for azole resistance. In three wells the agar was supplemented with itraconazole (4 mg/L), voriconazole (1 mg/L), or posaconazole (0.5 mg/L). The fourth well served as growth control. Growth was assessed after 24 and 48 hours of incubation and any growth on the agars supplemented with azoles was indicative of possible azole resistance. These isolates were further analysed at the Mycology Reference Laboratory (Radboud University Medical Center, Nijmegen, the Netherlands).
University Medical Centre, Nijmegen), including molecular identification, azole resistance phenotype and resistance genotype [3]. The minimum inhibition concentration (MIC) was determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution reference method [18]. Azole resistance was defined as a MIC-value>2 mg/L for itraconazole and/or voriconazole and/or a MIC-value>0.25 mg/L for posaconazole [18].

Microsatellite typing was retrospectively performed on available isolates by the Mycology Reference Laboratory (Nijmegen), as described previously [19]. In summary, six short tandem repeats (STR) were selected and amplified in two multiplex polymerase chain reactions (PCRs). The repeat numbers of the six markers were analysed by Bionumerics version 4.6, software (Applied Maths, Kortrijk, Belgium). The summed absolute distance between two multilocus variable-number tandem repeat analysis (MLVA)-typed isolates is the summed tandem-repeat differences (STRD) at six markers. Isolates with an STRD≤10 were defined as genetically related, irrespective of the number of differing loci. Clonal complexes were defined as by an STRD≤2 provided that isolates were single-locus variants or double-locus variants of one another.

Endpoints
The primary endpoint was the prevalence of IA by azole-resistant *A. fumigatus* at the ICU. Secondly, we investigated patient characteristics coinciding with resistant IA, such as pre-treatment with azoles, underlying illness, and IA probability according to EORTC/MSG criteria [15]. Moreover, 90-day mortality and cause of death were assessed. Furthermore, we compared ICU prevalence of IA by azole-resistant *A. fumigatus* with the prevalence in the same period in the rest of the hospital.

Data analyses
Descriptive statistics were used for primary analysis and assessment of clinical relevance. A formal risk assessment with multivariate analyses could only be performed in the presence of enough events, in order to prevent false positive results.

Results
During the study period, 197 prescriptions of voriconazole, c-AmB or L-AmB were identified in the ICU. Thirty-three patients received both voriconazole and c-AmB or L-AmB and appeared twice in the search. Twenty-eight patients were excluded; 21 patients received antifungal prophylaxis, and seven patients were treated for proven fungal infection due to yeast or fungi other than *A. fumigatus*. Consequently, a total of 136 patients were treated for suspected IA, representing 15 patients per 1,000 admissions at the ICU. A culture for *A. fumigatus* had been attempted for all 136 patients. Of these, 38 (28%) had a positive culture for *A. fumigatus* and were analysed further (Figure 1).

Azole resistance prevalence
Azole-resistant isolates were not recovered in 2010, but in the two following years a total of 10 culture-positive patients with resistant isolates were identified (Figure 2). Azole resistance was present in 10 of the 38 culture-positive patients (26%), which corresponds to 7% (10/136) of all patients receiving antifungal treatment in the ICU from January 2010 to December 2013, including culture-negative patients. In all other departments in the hospital, of all patients treated for suspected IA, *A. fumigatus* was cultured in 25 patients. Six of these patients had an azole resistant strain, yielding an azole resistance prevalence of 24% (6/25) (95% confidence interval (CI): 7–41%), which was seemingly comparable to that in the ICU.

Clinical characteristics of *Aspergillus fumigatus* culture positive patients in the intensive care unit
No differences were found in patient characteristics between the 10 patients with IA by azole-resistant *A. fumigatus* when compared with 28 patients with susceptible isolates (Table 1). Our low number of events (n=10) unfortunately prevented us from doing multivariable analysis.

Underlying disease
In the entire study group encompassing 38 patients with suspected IA in the ICU, 16 underwent a haematopoietic stem cell transplantation and four suffered from haematological malignancy, but were not yet transplanted (Table 1). Three patients had chronic immunosuppressive therapy for solid organ transplants. Other host factors included autoimmune disease (n=6), history of pulmonary disease (n=6: cancer, chronic obstructive pulmonary disease (COPD), irradiation) and the admission diagnoses of the remaining three patients were heart failure, acute liver failure, and severe disseminated infection with *Strongyloides stercoralis*.

Diagnosing invasive aspergillosis using international criteria
Of 28 patients with an azole-susceptible *A. fumigatus* infection, 15 were classified as probable IA, while proven IA was only established in two. Eleven of 28 patients with an azole-susceptible strain did not fulfil EORTC/MSG criteria of proven, probable or possible IA, most often due to lack of host factor, inconclusive CT scan or inability to perform a CT scan. Of 10 patients with an azole-resistant *A. fumigatus*, five patients remained unclassified according to the EORTC/MSG criteria; in three cases, CT scans showed unspecific changes and two patients were not stable enough to be transported to the CT scan, but did show bilateral pulmonary infiltrates on the chest X-ray. The other five qualified as probable IA. Overall 22 of 38 patients could be classified according to EORTC/MSG.

Except for one patient, who was on ‘Extra Corporeal Membrane Oxygenator’ (ECMO) therapy for heart
failure, applying the recently published ICU criteria of Blot et al. [16], allowed classification of all patients with an *A. fumigatus* positive culture. ECMO therapy hampers the assessment of *A. fumigatus*-related clinical signs and symptoms (fever, dyspnoea, worsening of respiratory insufficiency), since they are regulated by the device itself. According to this system, a total of two patients, both infected with an azole-susceptible strain, had proven IA. A further twenty-one patients with an azole-susceptible strain qualified as having putative IA as well as all 10 patients with azole-resistant *A. fumigatus*. Only four patients, all with an azole-susceptible *A. fumigatus* isolate, were classified as colonised with *Aspergillus* according to Blot’s criteria. For one of these (with a BAL), post-mortem results indicated angio-invasive aspergillosis. For the other three patients, difficult mechanical ventilation prohibited the performance of BAL. After intubation, a tracheal aspirate with a positive *A. fumigatus* culture was however available for these three patients. EORTC criteria would classify one of them as probable invasive pulmonary aspergillosis, while post-mortem findings in another were consistent with angio-invasive aspergillosis. In the fourth patient the diagnosis allergic broncho-pulmonary aspergillosis (ABPA) was made.

**Azole prophylaxis**
The relative amount of patients receiving prior treatment with azoles appeared higher in the group developing IA due to resistant *A. fumigatus*. The low number of events however prohibited a formal statistical risk assessment (Table 1).

**Microbiological characteristics of Aspergillus fumigatus culture-positive patients at the intensive care unit**

**Galactomannan in bronchoalveolar lavage fluids**
Overall of the 136 patients treated in the ICU on suspicion of IA, galactomannan testing was performed for 124, mostly on BAL fluids. In 82 of these 124 patients antigen testing was positive.
In 38 of the 136 patients *A. fumigatus* could be cultured. A BAL was not performed in five of the 38 culture-positive patients, since a lung biopsy was available for one patient, serum galactomannan was positive in another neutropenic patient and clinical condition did not allow interventions in three patients. In 33 patients who underwent BAL, 29 had samples positive by galactomannan testing.

**Time to detection of resistant strain**

In most of the 10 ICU patients found with a resistant strain, the resistant strain was cultured before admission, or shortly after admission at the ICU, whereby the time after ICU admission that it took to be aware of the resistance had a median 1 day and a range of 0 to 20 days. In the six patients with azole-resistant strains at the non-ICU departments, resistant strains could also be cultured shortly after hospital admission (median 4 days; range: 1–71 days) (Table 2).

**Resistence mechanisms and microsatellite typing**

A cyp51A-mediated azole resistance mechanism was found in all ICU patients (Table 2). Microsatellite typing was available for resistant isolates of seven patients (4 from ICU population, 3 from non-ICU departments), all cultured in 2013 (Figure 3). Isolates of unique patients belonged to identical clonal complexes, although for one patient the presence of two genetically unrelated strains was revealed. These strains differed also phenotypically, with isolates either susceptible or with varying degrees of resistance. Only two patients, one hospitalised in the ICU the other elsewhere, shared a genetically related isolate (Figure 3; STDR < 10), but we did not find an epidemiological link between these patients.

**Outcome**

The 90-day mortality in 10 patients with an IA by azole-resistant *A. fumigatus* was 100% (with 90% within 30 days after ICU admission), compared with 82% (n=23)
in the 28 patients with a suspected azole-susceptible IA. Given that among the latter, one patient on ECMO was neither classifiable by Blot nor EORTC/MSG methods, and one patient, who was classified as colonised according to Blot might have actually been colonised (since he was diagnosed with BCPA), the 90-day mortality in azole-susceptible IA-group would be 88% (23/26).

In the group of patients infected with azole-resistant strains, cause of death was IA in all patients, except one. This patient’s clinical course was most likely unfavourable due to acute liver failure after a very recent liver transplantation. Of 28 patients infected with azole-susceptible A. fumigatus, the cause of death was attributable to IA in seven patients, whereas five patients died of respiratory failure caused by multiple pathogens and 11 patients died of other causes than IA (heart failure, sepsis, liver failure).

In four of 10 patients infected with azole-resistant A. fumigatus, azole resistance was detected after the patient had deceased. Hence, only six of 10 patients with a resistant isolate were treated with L-AmB, of which four received treatment for over 2 weeks (Table 1).

Discussion

Our study shows a very high prevalence (10/38) of azole-resistant A. fumigatus in patients suspected of IA in the ICU of a university hospital in the Netherlands. This prevalence is even higher (10/33) among patients with putative or proven IA according to the Blot criteria, whereby patients classified as ‘colonised’ with A. fumigatus are excluded. Both prevalence estimates nevertheless appear much higher than those reported in two previously published large Dutch surveillance studies [1-3], where, irrespective of the clinical relevance of the culture result, all Aspergillus spp. isolates cultured from clinical samples in five university hospitals were routinely screened for the presence ofazole resistance. In the first study covering the 2007 to 2009 period, azole resistance was found in 4.6% (82/1,782) of A. fumigatus isolates [3], while in the second study, between 2009 and 2011, this increased to 6.8% (63/921) of such isolates [2].

Prevalence of azole resistance in specific patient groups has only been recently investigated. A published report from another academic centre in the Netherlands, revealed a high incidence ofazole resistance (16.2%) in 105 high-risk patients with IA, with an even higher value in the haematology population (25%) and 10% in ICU patients [20]. Similarly, a high azole-resistant rate of 29.6% was demonstrated in A. fumigatus isolates from haematological stem cell transplant patients in Germany [6]. Our study also shows high prevalence of resistance in other departments of our hospital.

Although no known risk factors for developing IA by azole-resistant A. fumigatus were apparent, one could speculate that patients with IA who are transferred to the ICU are those whose condition deteriorates despite voriconazole treatment in the hospital wards, and thus are more likely to carry a resistant strain. Resistance rates could be overestimated due to the use of antifungal therapy at the time BAL is performed, because azole-susceptible strains are less likely to grow under mold-active therapy. Furthermore, as tertiary stem cell and solid organ transplant centre, our patient population is probably not representative for most ICUs in other hospitals. The conclusions from this study, which are also based on small numbers of patients, therefore cannot be applied to other ICUs with a higher number of immunocompetent patients.

Recognition of IA and start of empirical treatment remains difficult at the ICU. First of all, diagnostic criteria for patients with suspected IA are suboptimal. As demonstrated in this study, EORTC/MSG consensus definitions for IA are not generally applicable for ICU-patients: only 22 of 38 culture-positive patients could be classified. Second, new categories of ICU patients susceptible to invasive pulmonary aspergillosis, are increasingly reported [11,12,21]. Since these new categories of ICU patients lack the host-factors defined by EORTC/MSG consensus definition, new criteria have been proposed for ICU-patients [11,16,21]. Blot et al. suggested a different algorithm that appears to be more suitable, although some patient categories at risk, for instance liver failure, COPD, and solid organ cancer, are not included in these diagnostic criteria [11,16]. When applying the criteria of Blot to our group of 38 ICU patients, 37 of the patients could be categorised, though in two patients, classified as ‘colonisation’, post-mortem findings were congruent with angio-invasive aspergillosis, and one would be diagnosed probable IA, if one would use EORTC/MSG criteria, illustrating the restraints of this scoring system. Hence, development of better clinical criteria for diagnosing IA, especially in ICU patients, is warranted [16].

Multiple mechanisms can be considered with regard to the development ofazole resistance in A. fumigatus. In our study we found a short interval from admission to detection of a resistant strain (median 3 days, mean 8 days; range: 0–71 days). Moreover, molecular typing, did not reveal genetically-related strains, suggesting different sources of infection, instead of an outbreak of azole-resistant A. fumigatus or patient-to-patient transmission. This could imply that acquisition of resistant strains could have occurred before hospital admission. Some reports suggest an increase of prevalence of resistant A. fumigatus strains in the environment [2,22]. The use of fungicides in the agricultural sector might contribute to this phenomenon [1]. Other reports suggest development ofazole resistance during therapy. A review regarding this subject reports a median time for development of resistance under therapy of 4 months, with a range of 3 weeks to 23 month [23]. In our study the number of events was too small to rule out prior treatment or prophylaxis with azoles.
Finally, other studies suggest the concomitant presence of both susceptible and resistant strains in patient samples [24], resulting in a selection of resistant strains during therapy. It is not unlikely that all of the mechanisms mentioned above contribute to an observed increase of resistance in patients with *A. fumigatus* infections, making it difficult to predict which patients are carriers of a resistant strain.

Additionally, identifying IA with azole-resistant *A. fumigatus* is still dependent on culture-positivity and technical facilities. From the 136 patients initially suspected of IA in the ICU, 124 underwent the galactomannan test, which was positive for 82 (66%). In contrast *A. fumigatus* could only be successfully cultured in 28% (38/136) of patients suspected of IA in the ICU. In this culture positive group BAL galactomannan was more frequently positive (29/33). Hence a negative culture may not necessarily mean the absence of IA.

<table>
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<tr>
<th>Patient number</th>
<th>Predisposing condition</th>
<th>EORTC classification</th>
<th>Time in days from admission to finding a culture of resistant <em>A. fumigatus</em></th>
<th>Underlying lung disease</th>
<th>Neutropenia at admission</th>
<th>Azole pre-treatment</th>
<th>MIC&lt;sup&gt;a&lt;/sup&gt; (mg/L)</th>
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ANCA: antineutrophil cytoplasm antibodies; COPD: chronic obstructive pulmonary disease; GOLD: Global Initiative for Chronic Obstructive Lung Disease; ICU: intensive care unit; EORTC: European Organization for Research and Treatment of Cancer; MIC: minimum inhibitory concentration; NA: not available; NSCLC: non-small cell lung carcinoma; SCT: stem cell transplant.

<sup>a</sup> The patient number is assigned consecutively within each patient population (i.e. ICU or non-ICU) so patients belonging to separate population groups but with the same number are different patients.

<sup>b</sup> MICs were evaluated for itraconazole (itra.), voriconazole (vori.), or posaconazole (posa.).

### Table 2

Characteristics of patients infected with azole-resistant *Aspergillus fumigatus* in a university hospital, Netherlands, 2010–2013 (n=16)

as a risk factor. Finally, other studies suggest the concomitant presence of both susceptible and resistant strains in patient samples [24], resulting in a selection of resistant strains during therapy. It is not unlikely that all of the mechanisms mentioned above contribute to an observed increase of resistance in patients with *A. fumigatus* infections, making it difficult to predict which patients are carriers of a resistant strain.

Currently, the presence of resistance can be established only in culture-positive patients. In culture-positive patients, detection of azole resistance is often delayed, since MIC-testing of *A. fumigatus* is not routinely performed in clinical microbiology laboratories. Reference methods such as the EUCAST method require a mature culture and testing results are available 5 to 7 days after the culture has become positive. Screening of isolates with four-well plates has been shown to be useful and gives results within 24 to 48 hours, but this method is not commercially available [1,3].

Direct molecular testing for azole resistance in patients with high clinical suspicion and a positive antigen testing of BAL fluid appears to be the most promising method for early detection of azole-resistant *A. fumigatus* [25-27]. However, there are several technical issues to be resolved, such as the presence of single copy gene mutations of the cyp51A gene limiting the sensitivity of PCR detection. The most frequent found azole resistance mechanism in *A. fumigatus* is a mutation in the target protein [2,5]. The cyp51A gene encodes lanosterol 14α-demethylase that catalyses a step in the biosynthetic pathway of ergosterol (an essential...
High resistance rates urge us to explore alternative (empirical) treatment strategies for IA [28]. These include first-line treatment with a polyene or with combination therapy. The clinical experience with these alternative treatment options is very limited, but animal models showed similar efficacy of L-AmB in IA by azole-susceptible and azole-resistant isolates, independent of the underlying resistance mechanism [29].

The combination of voriconazole and anidulafungin has also been shown to be effective [30]. MIC-guided high dosing of azoles is still under investigation, but is probably of limited use due to adverse events [31,32].

In conclusion, we observed very high rates ofazole resistance A. fumigatus in culture-positive ICU patients with IA and very high mortality rates among such patients. Generalising our results requires caution, though recent reports suggest that similar trends are observed among other patient groups [20,25]. There is an urgent need to identify molecular markers for resistance in A. fumigatus other than the cyp51A-gene in order to enable the development of rapid molecular tools. Development of adequate diagnostic criteria for early diagnosis and reliable classification remains a challenge in IA, especially in the ICU setting.

Conflict of interest

None declared.

Authors' contributions

Judith van Paassen contributed to design, acquisition of clinical data, data analysis, writing and revising of the manuscript, and takes responsibility for the integrity of the data and the accuracy of the data analysis, and serves a first author. Anne Russcher contributed to study design, acquisition of relevant microbiological data, data analysis and writing and revising the manuscript. Astrid in ‘t Veld - van Wingerden contributed to acquisition of relevant microbiological data, data analysis, and writing and revising the manuscript.

References


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