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Azole resistance in
Aspergillus: epidemiology
and surveillance

Vrijdag 16 september om
12:30 uur precies

U bent van harte welkom
bij deze plechtigheid en de
aansluitende receptie in de
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Explanation of the cover:
The background photograph is a microscopic view of azole-resistant *Aspergillus fumigatus* hyphae, recovered from pus from a patient with a kidney transplant. The *Aspergillus* isolate is projected over a picture of the globe, spreading out from a droplet in the middle of Europe nearby the Netherlands. It represents the global spread and threat of (knowledge and understanding of) resistant *Aspergillus fumigatus* around the world.


Lay-out by Corné van de Laar

Cover design by Jan van der Linden

The photograph on the cover was used with courtesy of Prof. dr. P.E. Verweij

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AZOLE RESISTANCE IN ASPERGILLUS: EPIDEMIOLOGY AND SURVEILLANCE

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op vrijdag 16 september 2016
om 12.30 uur precies

door
Jan Wilhelmsus Marinus van der Linden
geboren op 7 februari 1984
te Nijmegen
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Chapter 1:

General introduction.
Aspergillus.

In 1729 Aspergillus was first described by priest and biologist Antonio Michelli, who named the fungus because of its microscopic resemblance to an aspergillum, which is a tool used by priests for sprinkling holy water. Since then, over 180 species within its genus were identified based on differences in macro- and microscopic morphological features. At present, multilocus sequence-based phylogenetic analyses of mainly β-tubulin or calmodulin sequences have emerged as the primary tool for Aspergillus species identification. Since then, numerous new species have been proposed for the most prevalent Aspergillus sections including Fumigati, Flavi, Terrei, Nidulanti and Nigri.[1] Aspergillus section Fumigati now contains as many as 25 different species, 8 anamorphs and 17 teleomorphs, based on sequence-based identification.[2] Aspergillus is a saprophytic mould, found worldwide and one of the characteristics of its biological life-cycle is the high sporulating capacity in which it ubiquitously presents high concentrations of conidia (1-100 conidia m³) in the environment (Figure 1). These conidia are continuously inhaled by humans but are seldom pathogenic due to an efficient elimination by entrapment in airway mucus and ciliary beating and innate immune responses consisting of phagocytic activity and antifungal killing by alveolar macrophages.[3] Though, in immunocompromised patients and patient with underlying chronic pulmonary illnesses, Aspergillus species are capable of establishing a wide variety of diseases.[4]

Figure 1. Microscopic detail (100x) of Aspergillus on lactophenol cotton blue staining, showing conidiophores with conidia. With courtesy of H.A.L. van der Lee.

Aspergillus disease.

Many authors describe Aspergillus as ‘the multi-artist’ as it is able to cause a wide range of diseases (Figure 2).[5-10] Thirty-three Aspergillus species have been associated with human disease.[11] The term ‘aspergillosis’ refers to illness due to any species of Aspergillus, but A. fumigatus is the most formidable Aspergillus causative species in most regions in the world, followed by A. flavus. [4] However, the illnesses caused by different species of Aspergillus are clinically
indistinguishable. Besides this, the extent of hyphal invasion in an individual patient is generally not due to extrinsic factors, like an increasing concentration of airborne *Aspergillus* conidia, or intrinsic characteristics of the fungus, but depends mainly on patient-related risk factors rendering them immunocompromised. Persistent neutropenia in patients with malignancies, recipients of hematopoietic stem cell or solid organ transplants, functional deficiencies in phagocytic cells as seen in patients with primary immunodeficiencies, and patients receiving prolonged treatment with corticosteroids display the highest risk for developing invasive aspergillosis. Chronic pulmonary aspergillosis (CPA) is a clinical entity observed in both patients with chronic obstructive pulmonary disease and cystic fibrosis patients. Although, in both patient groups the differentiation between colonization and disease is cumbersome and at present it is not clear if the presence of *Aspergillus* species in the CF lung contributes to progressive lung disease.[12-14] Allergic bronchopulmonary aspergillosis (ABPA) is observed in up to 12% in CF patients and is seen sporadically in patients with asthma.[15] An aspergilloma is the development of a fungal mass (fungal ball) in a preexistent pulmonary cavity, caused by tuberculosis or other cavitating lung disorders (e.g. sarcoidosis). Over the past years, major advances in healthcare resulting in an increased number of immunocompromised patients have led to a four-fold increase in the incidence of life-threatening *Aspergillus* infections, with up to 12% in allograft hematopoietic stem cell transplant recipients.[16,17]

**Figure 2.** Clinical spectrum of *Aspergillus* diseases: the interaction of *Aspergillus* with the host. Adapted from Kosmidis C, Denning DW. Postgrad Med J 2015;91:403-10.[10]
(Angio-)invasive aspergillosis:
Airborne conidia of *A. fumigatus* overcome the primary immune defense mechanisms of the lung in the immunocompromised patient, where they germinate in the alveoli and produce hyphae invading the lung tissue.[3] Besides invasion of lung tissue (70%), dissemination may occur to other organs, especially brain, thyroid and kidneys in 9% of patients with *Aspergillus* disease.[18] Patients being most at risk of invasive disease are those with prolonged and severe neutropenia, glucocorticoid therapy, hematopoietic and solid organ transplantation, acute and chronic graft-versus-host disease, advanced AIDS and chronic granulomatous disease.[4,16,19-21] Incidence rates vary according to the underlying disease of the patient, and range from 0.4% to 14% in patients who undergo haematopoietic stem cell transplantation to 0.3% to 6.9% in patients with other causes of immunosuppression admitted to the ICU.[22,23] A recent French study showed a significant increase of invasive aspergillosis over the last few years (2001-2010) of 2.7% in patients with hematological malignancies and of 9.8% in patients who underwent haematopoietic stem cell transplantation.[24]

A prompt diagnosis of invasive aspergillosis is crucial, as early antifungal therapy corresponds with a better response.[25,26] However, establishing a timely diagnosis remains difficult due to non-specific symptomatology, late appearance of radiological signs and moreover the low sensitivity of microbiologic cultures (positive cultures in 25-50% of patients with invasive disease).[27] Furthermore, cultures become often positive when the disease is advanced and the fungal burden relatively high. Antigen detection in blood and broncho-alveolar lavage (BAL) fluid has become an important diagnostic tool in diagnosing invasive disease. Examples of modern diagnostics are galactomannan, which is a component of the *Aspergillus* cell wall, beta-D-glucan, which is positive in a broad range of fungi, and PCR-amplification of fungal DNA.[28-32] However, the best possible option for conclusive diagnosis is a positive culture from a normally sterile environment combined with histologic confirmation, but this requires invasive procedures, which are commonly not tolerated in patients who are extremely ill or have other contraindications such as thrombocytopenia. Newer diagnostic tools, like volatile organic compound detection in exhaled air, have become available, though their diagnostic value still needs to be investigated.[33]

Invasive aspergillosis is lethal in 85% of untreated patients, but through effective therapy the mortality rate has fallen to below 28.5%.[17,18,24,34,35]

Chronic airway invasive aspergillosis:
Chronic pulmonary aspergillosis (CPA) is a more indolent form of pulmonary aspergillosis and occurs in various histological forms, from simple aspergilloma, to chronic cavitary pulmonary aspergillosis and to chronic fibrosing pulmonary aspergillosis, both with and without aspergilloma’s. Unlike acute (angio-)
invasive aspergillosis, CPA occurs in apparently immunocompetent patients. In most patients their host defenses are in an impaired state due to conditions like diabetes mellitus, a connective tissue disorder, poor nutrition, chronic obstructive pulmonary disease, asthma or low dose corticosteroid therapy. Aspergilloma are classically observed in patients left with lung cavities after pulmonary tuberculosis. It is believed to have an enormous underestimated global burden. In 2007 an estimated amount of 372,000 patients developed CPA, of which 11,400 in Europe and 145,372 in South-East Asia. The estimated prevalence rate ranged from < 1 case per 100,000 in large western European countries and the United States of America to 42.9 per 100,000 in both the Democratic Republic of the Congo and Nigeria. CPA has a case fatality rate of 20–33% in the short-term and of 50% over a span of 5 years.[36]

Patients with aspergilloma are usually asymptomatic and mortality is low when compared to invasive disease, with a case-fatality rate of 29.5%.[18] Lethality is caused by severe hemoptysis from an aspergilloma affecting the pulmonary vasculature. Medical treatment for aspergilloma is often ineffective, whereas surgical resection of the infected lung cavity is the definite therapy. [37] ABPA or severe asthma associated with fungal sensitization (SAFS) are allergic manifestations of Aspergillus colonization which consist of a unique Th2-cell mediated immune response and can cause severe bronchial asthma exacerbations which may proceed to histologic forms of CPA and end-stage lung fibrosis.[38,39] The prevalence of ABPA in asthma patients may be as high as 13%.[40]

Last, chronic tracheobronchitis is seen in patients undergoing lung transplantation and is due to colonization of the anastomosis. This disease entity ranges from relatively mild tracheobronchitis with excess mucus production and inflammation to ulcerative tracheobronchitis with ulcers (often around the suture line) and extensive pseudomembranous tracheobronchitis.[41]

**Treatment of Aspergillus disease.**
Currently, there are 3 classes of antifungal drugs with activity to Aspergillus species (Table 1), and might be used for prevention or treatment of invasive aspergillosis.

**Polyenes**
Polyene agents bind to ergosterol of the fungal cell wall, alter its permeability and cause oxidative stress and thereby cause rapid cell death. Historically, amphotericin B has been the major antifungal drug used for treating aspergillus diseases, but currently has been replaced by other antifungals due to the frequent encounter of nephrotoxicity and other lipid-based formulations that became available that exhibit an improved tolerability.[42-49]
Azoles
The mold-active azole compounds are considered to be the backbone of treatment of Aspergillus diseases. In the two past decades four different azole compounds became available, being respectively itraconazole, voriconazole, posaconazole and isavuconazole. Triazoles bind with their nitrogen group to the heme group located in the center of the Cyp51A-protein and thereby block sterol 14-alfa-demethylase, which is an enzyme in the ergosterol pathway of the fungus. Besides this, the accumulation of sterol intermediates will cause toxic stress.[34,35,37,56-66]

Echinocandins
Echinocandins inhibit (1,3)-β-d-glucan synthesis and thereby inhibit hyphal-growth. It causes aberrant growth of hyphae at the apical tips, but absolute killing does not occur. Caspofungin is the longest serving echinocandin with micafungin and anidulafungin completing the arsenal of echinocandins in the recent years. They are very well tolerated and have few drug interactions, though currently cannot be recommended for primary therapy of invasive aspergillosis as phase II studies indicated a lower efficacy than can be expected with azole drugs.[67-70]

In-vitro susceptibility testing of Aspergillus species.
For determination or prediction of the susceptibility profile of the causative agent in Aspergillus diseases a variety of methods have been proposed in the last two decades.

Microbroth dilution methods
There are currently two reference methods for in vitro susceptibility testing of Aspergillus species: the method of the Clinical Laboratory Standards Institute (CLSI) and of the European Committee for Antimicrobial Susceptibility Testing (EUCAST). The methods both have a microbroth dilution format using RPMI-1640 medium. The minimum inhibition concentration (MIC) is read visually after 48 hours of incubation. The EUCAST-method has determined clinical breakpoints for several Aspergillus species-antifungal drug combinations, including those for A. fumigatus and itraconazole, voriconazole and posaconazole.[71-74]

Agar dilution method and e-test
Disk-based susceptibility testing is convenient, simple and economical. The optimal testing conditions for Aspergillus disk diffusion testing are known. Agar-based methods hold promise as simple and reliable methods for determining susceptibilities of filamentous fungi. The commercial e-test method is suitable for Aspergilli and is a reliable and reproducible method. The results correlate well with the CLSI methodology, though MIC reading of echinocandins against Aspergillus might be troublesome because of heavy growth within a discrete ellipse.[74]
<table>
<thead>
<tr>
<th>Antifungal group</th>
<th>Drug</th>
<th>Efficacy of treatment</th>
<th>Pharmacokinetics</th>
<th>Tolerability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyenes</td>
<td>Nebulized liposomal amphotericin B</td>
<td>Long-term administration of prophylaxis can be used for decolonization and preventing IPA in lung transplant recipients. It was also evaluated as an antifungal prophylaxis in patients with hematologic diseases, but needs further investigation.</td>
<td>After nebulization concentrations remain high for 14 days, at adequate concentrations for prophylaxis of IPA. No significant systemic absorption was detected and no effect was observed on respiratory function.</td>
<td>Bronchospasm and nausea are reported in a low frequency.</td>
<td>42-45</td>
</tr>
<tr>
<td></td>
<td>Amphotericin B deoxycholate (conventional amphotericin B)</td>
<td>Amphotericin B is approved to treat IPA. However, the use of conventional amphotericin B is limited by significant dose-dependent toxicity.</td>
<td>Available in intravenous form. Shows nonlinear pharmacokinetics, with higher than dose-proportional increases.</td>
<td>Fever, chills, headache, nausea and vomiting, rigors, respiratory compromise, electrolyte abnormalities (hypokalemia, hypomagnesemia, hypematrema, and metabolic acidosis) are reported, though nephrotoxicity occurs in 30% of patients.</td>
<td>46,47</td>
</tr>
<tr>
<td>Liposomal amphotericin B</td>
<td>Inferior to voriconazole as first line treatment of IPA, though considered a first line option for empirical therapy in neutropenic patients with fever at high risk of IPA. The reduced toxicity of the lipid formulations of amphotericin contributed to the overall improved outcome in comparative studies, allowing for the use of higher doses with better fungal killing and less side effects, though the lipid-associated preparations did not appear to provide a survival benefit over conventional amphotericin B deoxycholate.</td>
<td>Same as amphotericin B deoxycholate, but because of its small size and negative charge, it avoids substantial recognition and uptake by the mononuclear phagocyte system. Therefore, a single dose of results in a much higher peak plasma level than conventional amphotericin B deoxycholate and a much larger area under the concentration-time curve.</td>
<td>Same as amphotericin B deoxycholate, though significantly less frequent when given at normal dosages.</td>
<td>46-49</td>
<td></td>
</tr>
</tbody>
</table>

| Azoles          | Itraconazole                   | Proven to be effective as prophylaxis in CGD patients and in CF and asthmatic patients with ABPA. Furthermore it can be used as prophylaxis for invasive Aspergillus infections in neutropenic patients. | Available in oral and intravenous forms. The bioavailability is highly variable. It has poor penetration into the central nervous system. Therapeutic drug monitoring and measurement of serum concentrations are highly recommended. | Dose-related nausea and vomiting, likely due to the cyclodextrin used to solublize the medication are reported. High doses of the medication can have an aldosterone-like effect, manifested by hypokalemia, edema, and hypertension. Cases of congestive heart failure have been reported, and therefore caution should be used in patients with ventricular dysfunction. | 49-55     |

| Voriconazole    | Superior to (conventional) amphotericin B, showing a significant improved survival (71% versus 58%), and is approved for first-line therapy of IPA. In a single-center study with 405 hematopoietic stem cell transplantation patients with probable or proven invasive aspergillosis the widespread use of voriconazole since 2002 was associated with reduced risk of death in the univariate analyses. | Available in oral and intravenous forms. The oral form has a bioavailability of greater than 90%. Penetrates well into the central nervous system. Shows nonlinear pharmacokinetics in adults. Because of variable population pharmacokinetics and dose–response relationships, the narrow therapeutic window, and a clinically defined therapeutic range for clinical response, therapeutic drug monitoring is essential, especially in children, in whom clearance is linear and more rapid. | Available in oral and intravenous forms. | Visual disturbances, skin rashes, hallucinations, and hepatotoxicity are reported. Long-term use is associated with unexpected side effects that include alopecia, periostitis from fluoride excess, and development of skin cancers. | 34,35,37,56-58 |

Table 1. Antifungal agents with activity against *Aspergillus* species.
<table>
<thead>
<tr>
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<td>Fever, chills, headache, nausea and vomiting, rigors, respiratory compromise, electrolyte abnormalities (hypokalemia, hypomagnesemia, hypernatremia, and metabolic acidosis) are reported, though nephrotoxicity occurs in 30% of patients.</td>
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<td>Inferior to voriconazole as first line treatment of IPA, though considered a first line option for empirical therapy in patients with hematologic diseases. Liposomal preparations did not appear to provide a survival benefit over conventional amphotericin B deoxycholate. Same as amphotericin B deoxycholate, but because of its small size and negative charge, it avoids substantial recognition by the reticuloendothelial system. Plasma levels are lower than conventional amphotericin B deoxycholate and a much larger area under the concentration-time curve.</td>
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<td>Itraconazole</td>
<td>Proven to be effective as prophylaxis in CGD patients and in CF and asthmatic patients with ABPA. Furthermore it can be used as prophylaxis for invasive Aspergillus infections in neutropenic patients. Available in oral and intravenous forms. The bioavailability is highly variable. It has poor penetration into the central nervous system. Therapeutic drug monitoring and measurement of serum concentrations are highly recommended. Dose-related nausea and vomiting, likely due to the cyclodextrin used to solubilize the medication are reported. High ... heart failure have been reported, and therefore caution should be used in patients with ventricular dysfunction.</td>
<td>Visual disturbances, skin rashes, hallucinations, and hepatotoxicity are reported. Long-term use is associated with unexpected side effects that include alopecia, periostitis from fluoride excess, and development of skin cancers.</td>
<td>34,35,37,56-58</td>
</tr>
<tr>
<td></td>
<td>Posaconazole</td>
<td>Licensed for antifungal prophylaxis in hematological high-risk patients and for salvage therapy of invasive aspergillosis. Posaconazole is effective as prophylaxis in patients with myelodysplastic syndrome or acute myelogenous leukemia and patients with severe GVHD. There is no evidence for using posaconazole as primary therapy, although studies are ongoing with the intravenous formulation of the drug. Available in oral and intravenous forms. No serious side effects are reported, but headache, fever, nausea, vomiting, and diarrhea can occur. Rarely, hepatotoxicity and QT interval prolongation have been reported.</td>
<td></td>
<td>59-63</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>Superior to (conventional) amphotericin B, showing a significant improved survival (71% versus 58%), and is the most frequently used drug in the US. The widespread use of voriconazole since 2002 was associated with reduced risk of death in the univariate analyses. Available in oral and intravenous forms. The oral form has a bioavailability of greater than 90%. Penetrates well into the central nervous system. For consolidation therapy, therapeutic drug monitoring is essential, especially in children, in whom clearance is linear and more rapid.</td>
<td>Side effects of headache, rhinitis, nasopharyngitis, moderate diarrhea, nausea, and mild upper abdominal pain have been seen in healthy volunteer studies.</td>
<td></td>
<td>64-66</td>
</tr>
<tr>
<td></td>
<td>Isavuconazole</td>
<td>In vitro activity against most Aspergillus isolates and has also been shown to be effective in animal models of disseminated aspergillosis, though it is currently in Phase III clinical development for treatment of aspergillosis. Available in oral and intravenous forms. When compared to voriconazole it has shown low to moderate intersubject variability in serum levels.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echinocandins</td>
<td>Caspofungin</td>
<td>The treatment of invasive aspergillosis with caspofungin, micafungin, or anidulafungin as primary therapy has not been examined in a randomized controlled manner. In vitro the echinocandins have similar or better fungistatic activity than those of amphotericin B and the triazoles. The therapeutic efficacy of caspofungin is superior to amphotericin B and has demonstrated efficacy as empiric treatment of febrile neutropenia and salvage therapy for the treatment of invasive aspergillosis. Caspofungin is approved by the FDA as salvage therapy for invasive aspergillosis. None of the echinocandins is approved for the use in pediatric patients. Minimal effective concentrations of micafungin and anidulafungin are 2-10 fold lower than those for caspofungin. Synergistic/additive in vitro effects (in presence of serum) of echinocandins when combined with a polyene or azole have been observed, and it appears that mortality is reduced with combination therapy compared with monotherapy. Available in intravenous form. Distribute well into tissues, but poorly into the central nervous system and eye.</td>
<td>Phlebitis, headache, rash, pruritis, hypokalemia and elevated liver enzymes are reported. A higher rate of adverse reactions has been reported for caspofungin compared with micafungin and anidulafungin, though data with anidulafungin are limited.</td>
<td>67-70</td>
</tr>
<tr>
<td></td>
<td>Micafungin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anidulafungin</td>
<td></td>
<td></td>
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</tbody>
</table>
Molecular mechanisms and direct detection of resistance mechanisms. However, variable MIC profiles (for each isolate and for each antifungal drug) make it possible to miss resistance if screening is performed using a single antifungal.[75] A real-time polymerase chain reaction (PCR) assay to detect *Aspergillus* species in bronchoalveolar lavage (BAL) and sputum specimens was used in 2011. In sputum in 15 of 19 (78.9%) and 30 of 42 (71.4%) patients with ABPA and CPA *Aspergillus* was found. The CYP51A gene, known to be involved in resistance-development, was further amplified to detect key single-nucleotide polymorphisms (SNPs) and contained SNPs associated with triazole resistance in 55.1% of samples.[76] Further novel PCR assays to detect azole resistance-mediating mutations of *Aspergillus fumigatus* from clinical samples in neutropenic patients can be used to detect azole resistance and to optimize antifungal therapy in patients with aspergillosis.[77]

**Failure to treatment of Aspergillus disease.**

Resistance to azole compounds can be divided into two groups, with some *Aspergillus* (non-*fumigatus*) species long-time known to be intrinsically resistant and the other group, containing *Aspergillus fumigatus* sensu stricto isolates, which acquired a mechanism of resistance to azole drugs being recognized in recent years.

*Intrinsic resistance in Aspergillus isolates.*

Until recently, *Aspergillus* species identification was primarily based on macroscopic and microscopic morphology and sufficient to guide antifungal therapy. Recent taxonomic changes have led to the classification of cryptic *Aspergillus* species that exhibit in vitro susceptibility profiles that differ significantly from that of *Aspergillus fumigatus* sensu stricto.[78-80] For non-*fumigatus Aspergillus* isolates interpretative breakpoints are not available and it is not possible to classify specific species or isolates based on the MIC. The in vitro activity of antifungal agents of non-*fumigatus Aspergillus* isolates is often compared with *A. fumigatus*. However, the experience with MIC-testing of non-*fumigatus Aspergillus* isolates is limited and the growth characteristics or production of foliates may have impact on the MIC results or its reproducibility. Nevertheless, the recent taxonomic changes require us to reestablish the MIC distributions of the species complexes and their newly defined *Aspergillus* species. In addition to investigating the wild type MIC distributions, the role of the new sibling species in invasive fungal infections needs to be established.

*Acquired resistance in A. fumigatus.*

Resistance development in bacteria and viruses is a well-known phenomenon, but was unknown for fungal pathogens until 1994 when the first observations of fluconazole resistance in *Candida albicans* were reported.[81] In the late 1990s first reports emerged of *A. fumigatus* sensu stricto having acquired a
resistant phenotype.[82] Azole resistance has been found mainly in *A. fumigatus* following chronic azole therapy, and was thereby supposed to be acquired.[83-86] Patients receiving chronic azole therapy, mostly itraconazole, are those with aspergilloma in the lung or those with CPA or ABPA were vulnerable to resistance development.[84,85] In these patients resistance is most commonly observed in *A. fumigatus* and the isolates may be resistant to only itraconazole or exhibit a multi-azole or pan-azole resistant phenotype.[87] The phenotype depends on the underlying resistance mechanism, which commonly involves point-mutations in the *Cyp51A*-gene, the target for antifungal azoles.[71,84,85,88-94] This route of acquired resistance in *A. fumigatus* has long been perceived as a manageable problem as resistance development during azole therapy has been reported with only a very low frequency.[95-97]

In the Netherlands a second route of resistance development was suggested where clinical *A. fumigatus* isolates may have become resistant through environmental exposure to 14α-demethylase inhibitors (DMIs).[88,98-100] The first resistance mechanism that is believed to be of environmental origin consists of a substitution of leucine for histidine at codon 98 in the *cyp51A* gene in combination with a 34 base pair tandem repeat in the gene promoter (TR<sub>34</sub>/L98H).[98] The corresponding phenotype showed resistance to itraconazole, and intermediate susceptibility or resistance to voriconazole and/or posaconazole.[71,88] DMIs inhibit fungal Cyp51A and are abundantly used for crop protection and material preservation. Although *A. fumigatus* is not a target-organism for azole-fungicides, many of these were found to exhibit in vitro activity against *A. fumigatus*. Five DMIs, all from the triazole class, showed in vitro activity against wild type *A. fumigatus* but not against isolates harbouring the TR<sub>34</sub>/L98H resistance mechanism. Moreover, these five fungicides were shown to have a molecule structure that was highly similar to that of the clinically licensed triazoles, and models of the CYP51A-protein indicated that the binding to heme was most hindered in these fungicides when TR<sub>34</sub>/L98H was present.[99] Based on these observations it was hypothesized that *A. fumigatus* acquires resistance mechanisms through exposure to azole fungicides in the environment. Patients may inhale azole-resistant spores and subsequently develop azole-resistant aspergillosis. Due to the molecule similarity between certain azole fungicides and the medical triazoles, the activity of the latter is lost and patient fails to azole therapy.

**Epidemiology and surveillance of resistance.**

In 2009 and 2010 we described a case series of acute invasive aspergillosis caused by *A. fumigatus* resistant to medical triazoles in four patients (Table 2).[8,9] Three were diagnosed with CNS aspergillosis and this was the first time azole resistant CNS aspergillosis was reported. For the cultured isolates, the MICs of both itraconazole and voriconazole were above the concentrations achievable in plasma and cerebrospinal fluid, thereby precluding the use of these agents. All patients with CNS aspergillosis described in these papers died and, despite
### Table 2. Clinical characteristics in four patients with acquired azole resistant *Aspergillus fumigatus* disease.

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex/age</th>
<th>Specimen</th>
<th>MIC (mg/l)</th>
<th>Mutation(s)</th>
<th>Underlying disease</th>
<th><em>Aspergillus</em> disease</th>
<th>Previous treatment</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>M/31</td>
<td>Lung biopsy</td>
<td>&gt;16 8 0.5</td>
<td>TR34/L98H CGD, COPD</td>
<td>IPA</td>
<td>ITZ</td>
<td>Survived</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>F/11</td>
<td>Lung biopsy</td>
<td>&gt;16 16 2</td>
<td>TR34/L98H Precursor B cell lymphoblastic lymphoma</td>
<td>IPA and CNS aspergillosis</td>
<td>None</td>
<td>Died</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>F/42</td>
<td>Brain biopsy</td>
<td>&gt;16 8 0.5</td>
<td>TR34/L98H HIV, COPD</td>
<td>CNS aspergillosis</td>
<td>None</td>
<td>Died</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>M/64</td>
<td>Brain biopsy</td>
<td>&gt;16 16 0.5</td>
<td>TR34/L98H Colon carcinoma with liver metastases</td>
<td>CNS aspergillosis</td>
<td>None</td>
<td>Died</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

M; male, F; female, ITZ; itraconazole, VCC; voriconazole, POS; posaconazole, CGD; chronic granulomatous disease, COPD; chronic obstructive pulmonary disease, HIV; human immunodeficiency virus, IPA; invasive pulmonary aspergillosis, CNS; central nervous system.

the generally dismal outcome of CNS aspergillosis, azole resistance precluded prompt adequate antifungal therapy. Furthermore, all patients harbored the same TR34/L98H resistance mechanism and in none of the patients' apparent risk factors for azole resistance existed: 3 of the 4 patients were not exposed previously to mold-active antifungal azoles. This underlines our hypothesis that resistance might have developed through fungal adaptation to exposure to azole fungicides in the environment.

Our case-reports showed that azole resistance further complicates the management of invasive aspergillosis and should be considered as a cause of treatment failure. Investigation of the fungus culture collection of the Radboud University Medical Center indicated that azole resistance had emerged in 2000, as in over 650 isolates collected between 1994 and 2000 azole resistance was not found. However, since the first azole-resistant isolate was found in 2000, each year patients with azole-resistant isolates were found with an increasing frequency over time. Over 90% of azole-resistant *A. fumigatus* isolates harbored the TR34/L98H resistance mechanism, indicating that the environmental route of resistance selection was dominant compared with the patient route. The study also indicated that azole resistance was not a local problem as azole resistance was found in *A. fumigatus* isolates sent by other Dutch Centers to Nijmegen for in vitro susceptibility testing.[88] As these isolates were not collected systematically, there was a clear need to determine the epidemiology of azole resistance in patients at risk for *Aspergillus* diseases and to investigate if patient risk factors could be identified.
Until recently in vitro susceptibility testing was not performed routinely in many clinical microbiology laboratories, but due to new taxonomic insights in *Aspergillus* classification associated with the recognition of variability in susceptibility profiles, and the emergence of acquired resistance the necessity to determine susceptibility profiles of clinical isolates will increase.

**Objective of this thesis.**
The aim of this thesis is to investigate the frequency of azole resistance through surveillance of clinical *A. fumigatus* isolates. In addition to the epidemiology of azole resistance and distribution and trends in resistance mechanisms we also aimed to identify risk factors associated with azole-resistant *Aspergillus* disease. In *chapter 2* the characteristics of azole resistance selection through patient therapy was reviewed following a case of a patient who’s isolates changed from a wild type phenotype to an azole-resistant phenotype during azole therapy. In *chapter 3* the results of an epidemiological survey are presented. Through screening of clinical *A. fumigatus* isolates the frequency of azole resistance, underlying resistance mechanisms and clinical implications was analysed. In this study the TR*34/L98H* resistance mechanism was found to dominate in clinical azole-resistant *A. fumigatus* isolates. However, in December 2009 a new resistance mechanism was found, TR*46/Y121F/T289A*, associated with high-level voriconazole resistance. The migration of this resistance mechanism across Dutch hospitals and the recovery from the environment is described in *chapter 4*. Finally, a large international surveillance study was performed in order to determine the presence and frequency of azole resistance in 19 countries (*chapter 5*). In the last chapter (*chapter 6*) all the results are discussed in the view of our current knowledge and future prospects.
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28 | General introduction
Drawing made by a 14-year old boy who was suffering from T-cell lymphoma and invasive aspergillosis, with pulmonary and cerebral lesions.
Chapter 2:


Abstract
Nine consecutive isogenic *Aspergillus fumigatus* isolates cultured from a patient with aspergilloma were investigated for azole resistance. The first cultured isolate showed a wild type phenotype, but four azole-resistant phenotypes were observed in the subsequent eight isolates. Four mutations were found in the *cyp51A*-gene of these isolates, leading to the substitutions A9T, G54E, P216L, and F219I. Only G54 substitutions were previously proved to be associated with azole resistance. Using a Cyp51A homology model and recombination experiments in which the mutations were introduced into a susceptible isolate, we show that the substitutions at codons P216 and F219 were both associated with resistance to itraconazole and posaconazole. A9T was also present in the wild type isolate and thus considered a Cyp51A-polymorphism. Isolates harboring F219I evolved further into a pan-azole-resistant phenotype, indicating an additional acquisition of a non-Cyp51A-mediated resistance mechanism. Review of the literature showed that in patients who develop azole resistance during therapy, multiple resistance mechanisms commonly emerge. Furthermore, the median time between the last cultured wild-type isolate and the first azole-resistant isolate was 4 months (range 3 weeks to 23 months) indicating a rapid induction of resistance.

Introduction
*Aspergillus fumigatus* is able to cause a wide range of diseases, including allergic syndromes, aspergilloma, and invasive aspergillosis. Azoles play an important role in the management of *Aspergillus* diseases, but chronic treatment may cause the development of resistance, especially in patients with cavitary lesions, such as aspergilloma.[5,14,15] Infection with azole-resistant *A. fumigatus* is associated with a higher probability of treatment failure than infection due to isolates with a wild-type susceptibility,[14,29,31] which is supported by experimental models of aspergillosis.[6-9,17,18] Surveillance studies indicate that the prevalence of azole resistance varies widely between countries,[1,23,32] and there is increasing evidence that in addition to patient therapy, environmental exposure to azole compounds may be an important route of resistance development.[24,33] The most common mechanisms of resistance in *A. fumigatus* are modifications in the *cyp51A* gene.[20] *Cyp51A* encodes cytochrome P450 sterol 14α-demethylase and is the target for azole drugs. Azoles bind to the heme cofactor located in the active site of the *cyp51* encoded enzyme, thereby blocking ergosterol synthesis. Subsequent ergosterol depletion and the accumulation of unusual toxic sterols lead to inhibition of fungal growth.[22,34]

The objective of this study was to investigate the evolution of azole resistance development in a series of consecutive *A. fumigatus* isolates from a patient with aspergilloma. In addition, we reviewed the literature for other patients with development of resistant *A. fumigatus* disease.
Case report
A 48 year old female was diagnosed with tumor necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) in 2000. Seven years earlier, she had undergone bilobectomy of the right lung because of recurrent bacterial infections, including persistent *Mycobacterium avium* infection. In 2000, she was diagnosed with aspergilloma in a cavity of the right lung of the postoperative severely retracted right lung. Surgery was precluded due to the destroyed right lung and itraconazole (ITC) treatment was initiated. Adequate serum concentrations were observed. She was treated from April 2000 to November 2007; in 2007, a decision was made to stop ITC treatment, since her computed tomography scan and chest X rays repeatedly showed a complete resolution of the aspergilloma, with no sign of recurrence over 8 months. In addition, sputum repeatedly remained negative for *Aspergillus*. Over the next year, her condition was stable, until October 2008, when *Aspergillus* was again cultured from the sputum. She was started on ITC but was switch in weeks to voriconazole (VRC). That December, posaconazole (POS) was given to replace VRC because of an insufficient clinical response. However, after several months there was radiological evidence for relapse of the aspergilloma and for *Aspergillus* localization in the left lung. A subsequently recovered *A. fumigatus* isolate cultured from the sputum exhibited a multi azole-resistant phenotype, and treatment was switched to liposomal amphotericin B (L-AMB), which was subsequently given in combination with caspofungin (CAS). Despite one month of treatment with L-AMB and CAS, the patient had ongoing *Aspergillus* disease and died because of severe urosepsis caused by extended-spectrum-β-lactamase-positive *Escherichia coli*.

At autopsy, *Aspergillus* could not be identified in the left lung, despite extensive sampling. The right lung was severely fibrotic and showed a cavity filled with necrotic material and *Aspergillus* hyphae on microscopy, but no evidence for invasive disease was found.

Materials and methods

*Aspergillus fumigatus* isolates. From our patient, nine *A. fumigatus* isolates were obtained within a 10-month period (September 2008 to June 2009). All isolates were cultured from respiratory samples, and the primary colonies were subcultured on agar plates supplemented with azole compounds (ITC, 4 mg/liter; VRC, 1 mg/liter; POS, 0.5 mg/liter)[28] as part of a national surveillance study. Isolates that grew on these agar plates were further analyzed. A previously cultured *A. fumigatus* isolate from this patient with a wild-type phenotype was also selected for further investigation. The isolates were identified by macroscopic and microscopic examination and ability to grow at 48°C.

Susceptibility testing. A broth microdilution test was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference method.[27] ITC, POS, VRC, and amphotericin B (AMB) were assayed over a two-fold concentration range from 16 to 0.016 mg/liter. Visual readings
were performed with a reading mirror, and an endpoint of 100% inhibition was used to determine the MICs. For interpretation of the azole MICs, the proposed breakpoints were used.[32]

**Cyp51A sequence analysis and microsatellite genotyping.** To isolate DNA, isolates were cultured on Sabouraud agar slants. Conidia were harvested and added to 200 μl of breaking buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 2% Triton X-100, 1% sodium dodecyl sulfate, 1 mM EDTA, pH 8) with ~0.1g glass beads (diameters, 0.4-0.6 mm). After shaking by vortexing, conidia were incubated at 70˚C for 30 min while shaking. Then, 200 μl of phenol-chloroform-isoamyl alcohol (25:24:1) was added and samples were incubated for 5 min while they were shaken. After centrifugation for 5 min, the upper phase containing the DNA was transferred to a new tube. One microliter of DNA was used per PCR.

The cyp51A gene was amplified and subsequently sequenced as described previously.[25] The promoter region was amplified using previously described primers P-A7 and P-A5[19] and sequenced using the forward primer (P-A7). The cyp51B gene and promoter region were amplified and sequenced using primer sets 5'-CTTTATTTCACTCCGACA-3'/5'-ACGCGAATACCCAGAA-3' and 5'-GGAGACTGCACAACAGC-3'/5'-GGAACCAGTGGAAGACCA-3'. To detect mutations, the sequences were compared with the cyp51A and cyp51B sequences with GenBank accession numbers AF338659 and AF338660, respectively.

From all isolates as well as three unrelated control isolates, six microsatellite loci (STRAf3A, 3B, 3C, 4A, 4B, and 4C) were amplified as described before.[10] The sizes of the fragments were determined, and repeat numbers were assigned.[24] Genotypes consisting of the number of repeats for each of the six microsatellites were created for all isolates.

P. digitatum (CAD27793), P. digitatum Cyp51B (ADO85403), P. marneffei Cyp51B (EEA25858), Phanerochaete chrysosporium (ACI23621), Pneumocystis carinii (ABG91757), Podosphaera fusca (ACT56506), Saccharomyces cerevisiae (AAA34546), Scheffersomyces stipitis (ABN68111), Schizosaccharomyces pombe (CAA90803), Talaromyces stipitatus (EED21732), Trichosporon asahii (ADN44281), Ustilago maydis (CAAS8176), Venturia inaequalis (AAF71293), V. nashicola (CAC85409), Yarrowia lipolytica (CAG82748), and Zygosaccharomyces rouxii (CAR28109). The positions of amino acid substitutions present in the patient isolates as well as their equivalents in other fungi were pinpointed into the alignment.

Furthermore, the locations of the observed amino acid substitutions were investigated with the help of a recently published homology model of the A. fumigatus Cyp51A enzyme.[25]

**A. fumigatus transformations.** Transformation experiments were performed as described before.[26] Briefly, site-directed mutagenesis was used to introduce the point mutations into a cassette containing the cyp51A gene and promoter region together with a hygromycin selection marker gene. Mutagenic primers 5’-CTGGCAAGGGCTTTACTCTCATCAATTATGCTACCG-3’ / 5’-CGGTAGCATATATATTGATGAGAGTAAAGCCCTTGTCCAG-3’ (for P216L) and 5’-GCTTTACTCCCATCAATTTATGCTACCGTGGGCC-3’ / 5’-GGCCCACGGTAGCATATATTGATGGGAGTGAAAGC-3’ (for F219I) were used. Both cassettes containing either the P216L- or F219I-causing mutation were used for homologous gene replacement by electroporation in a cyp51A wild-type isolate. Recombinants were selected on hygromycin-containing medium and subcultured for further investigation. In addition, a cassette without any mutation in the cyp51A gene was incorporated by electroporation as a transformation control. The complete cassette was sequenced to confirm the presence of the mutation and to ensure that no other mutations were present. To confirm that only one copy of cyp51A was incorporated, Southern blotting was performed. The susceptibility profile of the recombinants, the transformation control, and the transformation recipient isolate were determined by the EUCAST broth microdilution test as described above.

**Literature review.** We reviewed the literature for cases with acquired azole resistance development in A. fumigatus isolates during azole therapy. Only patients from whom at least one isogenic susceptible isolate was obtained were included, because in these cases it is very likely that resistance was acquired during treatment and not obtained from the environment. Information on Aspergillus disease, age, sex, underlying disease(s), treatment regimen, the number of isolates, the time needed for resistance to develop, and the resistance mechanisms observed was collected.
Results

Characterization of patient isolates. Results of susceptibility testing of the nine isolates obtained from our patient are shown in Table 1, together with Cyp51A substitutions and the number of repeats for each microsatellite maker. Microsatellite typing showed identical genotypes for all isolates, indicating that the collection of isolates was isogenic. The unrelated control isolates showed aberrant genotypes (data not shown).

The first isolate showed a wild-type susceptibility phenotype, while all other isolates showed an ITC MIC of >16 mg/liter. VRC MICs varied between 0.5 and 8 mg/liter, and POS MICs were between 0.063 and >16 mg/liter. In total, four different azole susceptibility profiles were observed. cyp51A sequence analysis showed a conversion of alanine (A) to threonine (T) at codon 9 (codon 9 was changed from GCC to ACC) in all isolates. All eight isolates with a non-wild-type azole susceptibility profile had an additional point mutation in the cyp51A gene. Six isolates showed a point mutation resulting in the substitution of phenylalanine (F) for isoleucine (I) at codon 219 (change of codon 219 from TTT to ATT). One isolate contained a mutation at codon 216 (changed from CCC to CTC), resulting in the replacement of proline (P) by leucine (L). The last isolate contained a point mutation leading to the substitution of glycine (G) for glutamic acid (E) at codon 54 (GGG was changed to GAG). No mutations in cyp51B were observed in any of the nine isolates.

Table 1. Isolates obtained from the patient suffering from pulmonary aspergilloma.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Date of isolation (day-mo-yr)</th>
<th>Specimen</th>
<th>Cyp51A substitution</th>
<th>MIC (mg/liter)</th>
<th>Microsatellite no. of repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ITC</td>
<td>3A</td>
</tr>
<tr>
<td>v74-61</td>
<td>29-9-2008</td>
<td>Sputum</td>
<td>A9T</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>v76-03</td>
<td>17-11-2008</td>
<td>Sputum</td>
<td>A9T, F219I</td>
<td>&gt;16</td>
<td>1</td>
</tr>
<tr>
<td>v77-41</td>
<td>17-12-2008</td>
<td>Sputum</td>
<td>A9T, P216L</td>
<td>&gt;16</td>
<td>1</td>
</tr>
<tr>
<td>v79-63</td>
<td>25-2-2009</td>
<td>Sputum</td>
<td>A9T, F219I</td>
<td>&gt;16</td>
<td>8</td>
</tr>
<tr>
<td>v80-28</td>
<td>9-3-2009</td>
<td>Sputum</td>
<td>A9T, F219I</td>
<td>&gt;16</td>
<td>8</td>
</tr>
<tr>
<td>v80-55</td>
<td>19-3-2009</td>
<td>Sputum</td>
<td>A9T, F219I</td>
<td>&gt;16</td>
<td>8</td>
</tr>
<tr>
<td>v82-58</td>
<td>16-5-2009</td>
<td>Sputum</td>
<td>A9T, F219I</td>
<td>&gt;16</td>
<td>4</td>
</tr>
<tr>
<td>v83-11</td>
<td>5-6-2009</td>
<td>Sputum</td>
<td>A9T, F219I</td>
<td>&gt;16</td>
<td>4</td>
</tr>
<tr>
<td>v83-14</td>
<td>7-6-2009</td>
<td>BAL</td>
<td>A9T, G54E</td>
<td>&gt;16</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Cyp51 sequence alignments and homology model. The Cyp51A amino acid substitutions observed in the patient isolates were studied in more detail. Cyp51 sequence alignments (Figure 1) showed that residue A9 is not well conserved. In contrast, G54, P216, and F219 are highly conserved within the fungal kingdom.
Figure 1. Alignment of fungal Cyp51 proteins. In case Cyp51 was specified as either Cyp51A or Cyp51B, this is indicated after the name of the fungus. The positions of *A. fumigatus* A9, G54, P216, and P219 are indicated in black; aligned positions in the other fungal species are indicated in gray. For each of the amino acids we are interested in, only 10 amino acids of surrounding sequence is added to the alignment. Other surrounding sequence is indicated by ***. The *A. flavus* Cyp51B sequence was relatively short compared to the other sequences. The alignment of *A. flavus* Cyp51B started at the position of residue 55 in *A. fumigatus* Cyp51A, so there is no comparable amino acid for *A. fumigatus* A9 and G54 in *A. flavus* Cyp51B.
Only *C. posadasii* showed a different amino acid at the position similar to *A. fumigatus* P216.

Because A9T is not well conserved and is also observed in the susceptible patient isolate and G54E is already described as being a cause of azole resistance [11], we focused on the P216L and F219I substitutions. To check for the position of these amino acids in the Cyp51A enzyme, P216L and F219I were pinpointed into a Cyp51 homology model. The model showed that both amino acids were located close to the opening of one of the two ligand access channels of the Cyp51 protein (Figure 2).

![Figure 2. Locations of residues P216 and F219 indicated in the Cyp51A homology model. The green tubes represent the two ligand access channels leading to the heme center of the enzyme.](image)

**A. fumigatus** recombinants. *A. fumigatus* recombinants were constructed with either the P216L or the F219I substitution. None of the recombinants had additional mutations in *cyp51A*, the promoter region, the 3’ region, or the hygromycin selection marker gene. Southern blotting confirmed that integration occurred at a single chromosomal locus in all recombinants (data not shown). As shown in Tables 1 and 2, the P216L recombinant exhibited a susceptibility phenotype similar to that of patient isolate v77-41, with resistance to ITC and

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Base change in <em>cyp51A</em></th>
<th>MIC (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Codon 216</td>
<td>Codon 219</td>
</tr>
<tr>
<td>Transformation recipient isolate</td>
<td>CCC</td>
<td>TTT</td>
</tr>
<tr>
<td>Transformation control</td>
<td>CCC</td>
<td>TTT</td>
</tr>
<tr>
<td>Recombinant P216L</td>
<td>CTC</td>
<td>TTT</td>
</tr>
<tr>
<td>Recombinant F219I</td>
<td>CCC</td>
<td>ATT</td>
</tr>
</tbody>
</table>

*a* Bold indicates the base change.

Table 2. MICs of recombinants with either the P216L or F219I substitution compared to those of the recipient isolate and the transformation control isolate.
an elevated MIC of POS. The phenotype of the F219L recombinant was similar to that of patient isolate v76-03 but different from the phenotypes of five other patient isolates with the F219I substitution (v79-63, v80-28, v80-55, v82-58, and v83-11) that were cultured later in the course of the disease.

**Previously described cases of acquired azole resistance.** Including the present case, eight cases with acquired azole resistance caused by *A. fumigatus* have been described (Figure 3). From each patient, between two and nine isogenic or nearly isogenic isolates of *A. fumigatus* were obtained, and the first isolate(s) showed a wild-type azole susceptibility phenotype. Except for patient 7, the *cyp51A* gene was sequenced and mutations at codons G54 (8 isolates from 5 patients), M220 (2 isolates from 2 patients), G448 (2 isolates from 2 patients), P216 (1 isolate), and F219 (6 isolates) were found. In five out of these seven cases, multiple resistance mechanisms emerged (patients 1, 3, 5, 6, and 8). All patients had azole exposure before the identification of the first resistant isolate. The antifungals used for therapy included ITC in two patients (patients 1 and 6), and in all other patients, multiple antifungal treatments had been used (Figure 3), mainly because of the development of resistance. The patient that we present

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**Figure 3.** Reported cases of acquired azole resistance in *A. fumigatus*. [2,3,5,6,14] The treatment schedules of all eight patients are indicated with bars, and the *A. fumigatus* isolates obtained from the patients are indicated with triangles. The corresponding resistance mechanisms are also indicated. wt, azole-susceptible wild-type isolate; ?, resistant isolate without any *cyp51A* mutations; ND, *cyp51A* sequence not determined; ITC, itraconazole; VRC, voriconazole; CAS, caspofungin; POS, posaconazole; AMB, amphotericin B (in various formulations). In the isolates marked with an asterisk, the F219L resistance mechanism was found in *cyp51A*. However, this isolate continued to evolve further azole resistance by an additional and yet unknown non-*cyp51A*-related resistance mechanism. Information regarding the treatment of patients 1, 2, and 3 was kindly provided by the author (S. Howard, personal communication).

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here also underwent multiple therapy changes, as described above. The time between the last culture of an isolate with a wild-type susceptibility phenotype and the first with an azole-resistant phenotype varied between 3 weeks and 23 months (median, 4 months).

Discussion
Azole resistance has been reported to emerge in patients with chronic pulmonary aspergillosis and pulmonary aspergilloma during azole therapy. Here, we describe a patient with pulmonary aspergilloma from whom isogenic azole-resistant *A. fumigatus* isolates with various resistance phenotypes were cultured during azole therapy. The resistant isolates showed different mutations in the *cyp51A* gene resulting in G54E, P216L, and F219I substitutions. In addition, all isolates contained a mutation leading to the A9T substitution. It is unlikely that this mutation will have an impact on azole susceptibility because the sensitive isolate also contained the A9T substitution and A9 is not well conserved among fungal species. Mutations at codon G54 are already known to be associated with azole resistance in *A. fumigatus* [5,11,12,16,21] and are even mentioned to be hot-spot mutations [13] because mutations at this site are commonly found in azole-resistant isolates. Clinical and laboratory-induced mutant *A. fumigatus* isolates with the G54E substitution in Cyp51A usually show the following susceptibility profile: ITC MIC, >8 mg/liter; VRC MIC, 0.25 to 0.5 mg/liter; and POS MIC, 1 mg/liter. [11,12,16] This profile is consistent with that of the isolate obtained from our patient. The Cyp51A P216L substitution was described before in a patient with chronic cavitary pulmonary aspergillosis with aspergilloma treated with ITC. [14] However, this substitution is not yet confirmed to be a cause of resistance by the construction of recombinants. To our knowledge, the F219I substitution has not been described before.

Sequence alignments revealed that residues P216 and F219 are conserved within the fungal kingdom, and the Cyp51A homology model showed that they are both located close to the opening of the ligand access channel, suggesting that these residues are important. As the channels are thought to be used by azole compounds to enter the active site of the protein, mutations at codons P216 and F219 might affect the docking of azole molecules. Previously, docking of azoles in a homology model of *A. fumigatus* Cyp51A showed an interaction of P216 and several other closely situated residues with POS but not with VRC, [34] indicating that mutations in this region might play a role in POS resistance. We confirmed the Cyp51A P216L and F219I substitutions to be the cause of azole resistance by the generation of recombinants in which the wild-type *cyp51A* gene was replaced by a *cyp51A* cassette containing either one of the two mutations. This resulted in resistance to ITC and POS, while recombinants remained susceptible to VRC. For P216L, this was in agreement with the phenotype of the clinical isolate, but for F219I, only one of six clinical isolates harboring this substitution showed a similar phenotype. For the remaining F219I
isolates, the VRC MIC had increased from 1 mg/liter to 8 mg/liter and the POS MIC had increased from 0.5 mg/liter to >16 mg/liter. As no additional cyp51A mutations were found in these five isolates, we assume that a second resistance mechanism not related to the cyp51A gene had evolved during POS treatment. This is reasonable because resistance mechanisms other than cyp51A mutations have been reported before in A. fumigatus.\[2,4,14\]

Review of the literature revealed seven other cases of aspergillosis caused by A. fumigatus in which azole resistance emerged during azole therapy. Because we selected only for cases in which at least one isogenic isolate with a susceptible phenotype was obtained, it is very likely that resistance was induced in the patient. The patients were probably initially colonized or infected with a susceptible isolate, and over time, through azole exposure, resistance developed in this initial isolate. In one case, the resistance mechanisms were not determined, but in five of the remaining seven cases (71%), at least two resistance mechanisms emerged, indicating that different evolutionary processes within one patient might result in independent adaptation of the fungus toazole exposure. Furthermore, in the case described here, resistance might also have accumulated sequentially as one of the resistant isolates further evolved to become multiazole resistant.

All patients were diagnosed with aspergilloma and chronic pulmonary aspergillosis. It was previously suggested that in the case of aspergilloma or cavitary aspergillus disease, the fungus is able to undergo multiple generations in the patient by the asexual way of reproduction. Sporulation (in the lung) as opposed to hyphal growth may be important to facilitate the expression of the azole-resistant phenotype; hyphal growth is typically found in acute invasive aspergillosis.\[33\]

Our literature review showed that the median time between the last cultured wild-type isolate and the first azole-resistant strain was only 4 months, indicating that resistance can be induced soon after initiating treatment. We have to address that two of the patients (patients 4 and 8; Figure 3) receivedazole treatment for several years before the last cultured wild-type isolate was obtained. It is therefore possible that under azole pressure a preceding event unrelated to cyp51A occurred in the wild-type isolate and that the event subsequently resulted in the rapid acquisition of the resistance mutations. From every clinical sample, only one colony is usually subcultured, stored, and subjected to susceptibility testing even when more colonies grow. Therefore, it cannot be excluded that the resistance mechanisms found persisted for a longer time. Moreover, additional resistance mechanisms may have been found when multiple colonies had been tested. Furthermore, patients are not usually regularly sampled for the presence of fungi, so isolates cultured could have already been present in the patient for a long time. Although it is now shown that azole resistance can be induced in the patient within a relatively short period of time, the questions of which proportion of azole-treated patients with aspergilloma
or other cavitary lung lesions develop azole resistance and whether specific risk factors for resistance development can be identified remain.

**Acknowledgments**
We thank Anthonius J. M. M. Rijs for susceptibility testing of the patient isolates and Anna Karawajczyk for her assistance with the Cyp51A homology model.

**References**


27. Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID


Drawing made by a 16-year old boy who was suffering from acute myeloid leukemia and invasive aspergillosis, complicated by hemoptysis and pneumomediastinum.
Chapter 3:


Abstract
The prevalence and spread of azole resistance in clinical *Aspergillus fumigatus* isolates in the Netherlands are currently unknown. Therefore, we performed a prospective nationwide multicenter surveillance study to determine the effects of resistance on patient management strategies and public health. From June 2007 through January 2009, all clinical *Aspergillus* spp. isolates were screened for itraconazole resistance. In total, 2,062 isolates from 1,385 patients were screened; the prevalence of itraconazole resistance in *A. fumigatus* in our patient cohort was 5.3% (range 0.8%–9.5%). Patients with a hematologic or oncologic disease were more likely to harbor an azole-resistant isolate than were other patient groups (p<0.05). Most patients (64.0%) from whom a resistant isolate was identified were azole naive, and the case-fatality rate of patients with azole-resistant invasive aspergillosis was 88.0%. Our study found that multiazole resistance in *A. fumigatus* is widespread in the Netherlands and is associated with a high death rate among patients with invasive aspergillosis.

Introduction
Azoles are the primary class of antifungal agents used for evidence-based treatment and prevention of *Aspergillus* spp. diseases.[1] Azoles are the only class of mold-active agents that can be used orally, and voriconazole and posaconazole have improved the survival of patients at risk for invasive aspergillosis.[2-5] However, recent reports describe the emergence of acquired resistance of *Aspergillus* spp. to azole compounds.[6-16] Azole resistance may develop in patients with cavitary lung lesions, such as aspergilloma, or in patients with allergic bronchopulmonary aspergillosis who are treated with mold-active azoles, most notably, itraconazole.[7,17] In these patients, resistance is most commonly observed in *Aspergillus fumigatus*, and the isolates may be resistant to only itraconazole (ITZ) or exhibit a multi-azole- or pan-azole-resistant phenotype. The phenotype depends on the underlying resistance mechanism, which commonly involves point mutations in the *cyp51A*-gene, the target for antifungal azoles.[6-8,10,11,17-21] A wide range of mutations was found in azole-resistant *Aspergillus* spp. isolates that were cultured from clinical samples from patients treated with azoles, and individual azole-resistant *Aspergillus* spp. colonies harbored different resistance mechanisms.[7] In the Netherlands, a highly dominant resistance mechanism was found in isolates from epidemiologically unrelated patients.[6,9] The dominant resistance mechanism consisted of a substitution of leucine for histidine at codon 98 of the *cyp51A*-gene in combination with a 34-bp tandem repeat in the promoter region of this gene (TR/L98H).[6,11] The corresponding phenotype showed resistance to itraconazole and intermediate susceptibility or resistance to voriconazole, posaconazole, or both.[6,20] TR/L98H isolates were recovered from azole-treated and azole-naïve patients with *Aspergillus* spp. diseases.[6,9,10,22-24] Azole resistance may be associated with a high probability of azole treatment failure, a possibility that is supported by preclinical evidence.[7,9,22-26]
On the basis of the above-mentioned observations, we hypothesized that resistance might have emerged as a consequence of exposure of *A. fumigatus* to azole fungicides in the environment.[27] Preliminary studies indeed showed that *A. fumigatus* isolates harboring TR/L98H could be recovered from the environment.[28,29] Furthermore, TR/L98H isolates were cross-resistant to certain azole fungicides.[27,28]

The spread and prevalence of azole resistance in clinical *A. fumigatus* isolates are unknown. Various studies in the Netherlands that have analyzed *Aspergillus* spp. culture collections indicate that the prevalence ranges from 1.8% to 12.8%.[6,30,31] Obtaining insight into the spread of azole resistance is essential for determining the implications of resistance for patient management strategies and public health. We therefore conducted a prospective surveillance study in 7 university medical centers in the Netherlands.

**Methods**

**Study Design.** All *Aspergillus* spp. isolates cultured from clinical samples that were processed in the 7 university medical microbiology laboratories were routinely screened for the presence of azole resistance, irrespective of the clinical relevance of the culture result. The University Medical Centers are located in 7 different cities in the Netherlands, and clinical isolates were screened during a 20-month period (June 2007–January 2009). Patient data were recorded in a Web-based database. An online questionnaire was completed for every collected isolate. The questionnaire included questions about isolate characteristics (species identification and date of isolation) and patient characteristics (age, sex, and underlying disease).

**Screening ITZ Agar Slants.** *Aspergillus* spp. colonies that grew in primary cultures were subcultured on Sabouraud agar slants supplemented with 4 mg/L of ITZ and incubated at 35°C–37°C. The colonies' ability to grow on the ITZ agar slants was assessed after 48 hours of incubation. For every isolate that was able to grow on the ITZ agar slants (ITZ positive), the primary culture isolate was sent to the Radboud University Nijmegen Medical Center for further analysis. Isolates that failed to grow on the ITZ agar slants (ITZ negative) were not analyzed. However, for all isolates that were screened, the online questionnaire was completed. At the screening sites, the *Aspergillus* spp. isolates were identified to species level by conventional methods, i.e., thermodurality and macroscopic and microscopic assessment of culture morphologic features.

**Proficiency Testing.** The ITZ agar slants were prepared at the Radboud University Nijmegen Medical Center and distributed to the other medical microbiology laboratories. Each center was provided with a protocol and a set of 6 *A. fumigatus* isolates. Four isolates were resistant to ITZ (MIC >16 mg/L), while 2 were susceptible (ITZ MICs 0.125 and 0.25 mg/L). The centers were blinded for the resistance profiles and were asked to determine the ability of the isolates to grow on the ITZ agar slants.
Analysis of ITZ-positive Isolates. Every ITZ-positive isolate was analyzed for certain phenotypic and genotypic features. The phenotypic analysis included the morphologic features of the isolate and susceptibility testing according to established standards[32] by using a broth microdilution format. MICs were determined for ITZ, voriconazole, and posaconazole. Genotypic analyses were performed by complete sequencing of the *cyp51A* gene by using the reference sequence of strain no. AF338659 from GenBank.

For the confirmed ITZ-positive isolates, additional patient data that includedazole exposure <3 months before the date of isolation of the resistant isolate and the presence of *Aspergillus* spp. disease were requested from the treating physician. Patients with cancer and invasive aspergillosis were classified according to the revised European Organization for Research and Treatment of Cancer/Mycoses Study Group definitions.[33] In addition, patient data were collected on treatment and outcome 3 months after diagnosis.

Statistical Analysis. Statistical analyses were performed by using SPSS version 17.0 (IBM, Somers, NY, USA). Analyses consisted of χ2 tests and a calculation of the κ coefficient.

Results

Proficiency Testing. Four of 6 control *A. fumigatus* isolates were resistant to ITZ and expected to grow on the ITZ agar slants, while the remaining 2 isolates were ITZ susceptible and should not grow on the ITZ agar slants. The 4 isolates with the azole-resistant phenotype harbored the TR/L98H resistance mechanism. For 3 ITZ-positive and the 2 ITZ-negative isolates, 100% agreement was found between the 7 centers. For 1 ITZ-positive isolate, the assessment of growth on the ITZ agar slant was incorrect from 3 centers; this isolate was a slowly sporulating *A. fumigatus* isolate. The κ coefficient calculated to assess the reproducibility of the method was 0.81.[34]

Mycology. During June 2007 through January 2009, we screened 2,062 *Aspergillus* spp. isolates from 1,385 patients for azole resistance using the ITZ agar slants. Most isolates were identified as *A. fumigatus* (1,792/2,062 [86.9%]) (Table 1). For 50 (2.5%) *Aspergillus* spp. isolates, species identification was not done. Most *Aspergillus* spp. isolates were isolated from respiratory samples; 1,461 of 2,062 (70.9%) were from sputum cultures, and 60 (2.9%) were from cultures derived from clinical specimens obtained from sterile sites (i.e., tissue specimens obtained through invasive procedures or at autopsy) (Table 1).

Ninety isolates were able to grow on the ITZ agar slants and were sent to the Radboud University Nijmegen Medical Center for further analyses. In vitro susceptibility testing showed that for 84 (93.3%) of 90 ITZ-positive isolates, the MIC of ITZ was >2 mg/L, which was considered resistant.[20] Most ITZ-positive isolates also exhibited non-wild-type susceptibility profiles to voriconazole and posaconazole. A resistant phenotype for voriconazole (>2 mg/L) and posaconazole (>0.5 mg/L) was observed in 67/84 (79.8%) and 14/84 (16.7%) of ITZ-positive isolates, respectively. An intermediate susceptibility profile (2 mg/L for
Table 1. Characteristics of screened susceptible and resistant isolates of *Aspergillus* spp., the Netherlands, 2007–2009*

<table>
<thead>
<tr>
<th>Source and species</th>
<th>No. (%) susceptible, n = 1,978</th>
<th>No. (%) resistant, n = 84</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum</td>
<td>1,397 (70.6)</td>
<td>64 (76.2)</td>
</tr>
<tr>
<td>Ear swab</td>
<td>176 (8.9)</td>
<td>3 (3.6)</td>
</tr>
<tr>
<td>BAL fluid</td>
<td>97 (4.9)</td>
<td>6 (7.1)</td>
</tr>
<tr>
<td>Bronchus secretion</td>
<td>66 (3.3)</td>
<td>2 (2.4)</td>
</tr>
<tr>
<td>Throat/nasal swab</td>
<td>66 (3.3)</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Tissue</td>
<td>55 (2.8)</td>
<td>5 (6.0)</td>
</tr>
<tr>
<td>Skin swab/nail</td>
<td>38 (1.9)</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Mouth wash</td>
<td>26 (1.3)</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Pus/wound swab</td>
<td>16 (0.8)</td>
<td>1 (1.2)</td>
</tr>
<tr>
<td>Bronchial wash</td>
<td>11 (0.6)</td>
<td>0</td>
</tr>
<tr>
<td>Feces</td>
<td>8 (0.4)</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>22 (1.1)</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>No. isolates</th>
<th>Mutations in the Cyp51A gene</th>
<th>Median MIC, mg/L (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fumigatus</em></td>
<td>1,710</td>
<td>TR/L98H†</td>
<td>&gt;16 (16–&gt;16)</td>
</tr>
<tr>
<td><em>A. flavus</em></td>
<td>98</td>
<td>G54W†</td>
<td>&gt;16 (16–16)</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>52</td>
<td>P216L†</td>
<td>&gt;16 (16–16)</td>
</tr>
<tr>
<td><em>A. terreus</em></td>
<td>35</td>
<td>F219I†</td>
<td>&gt;16 (16–16)</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>14</td>
<td>None</td>
<td>&gt;16 (16–16)</td>
</tr>
<tr>
<td><em>A. versicolor</em></td>
<td>13</td>
<td>None</td>
<td>&gt;16 (16–16)</td>
</tr>
<tr>
<td><em>A. glaucus</em></td>
<td>6</td>
<td>None</td>
<td>&gt;16 (16–16)</td>
</tr>
<tr>
<td>Unknown</td>
<td>50</td>
<td>None</td>
<td>&gt;16 (16–16)</td>
</tr>
</tbody>
</table>

*BAL, bronchoalveolar lavage.

Table 2. Characteristics of itraconazole-positive *Aspergillus fumigatus* isolates, the Netherlands, 2007–2009*

<table>
<thead>
<tr>
<th>No. isolates</th>
<th>Mutations in the Cyp51A gene</th>
<th>Itraconazole</th>
<th>Voriconazole</th>
<th>Posaconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>TR/L98H†</td>
<td>&gt;16 (16–16)</td>
<td>8 (1–16)</td>
<td>0.5 (0.25–2)</td>
</tr>
<tr>
<td>1</td>
<td>G54W†</td>
<td>&gt;16</td>
<td>0.5</td>
<td>&gt;16</td>
</tr>
<tr>
<td>1</td>
<td>P216L†</td>
<td>16</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>F219I†</td>
<td>&gt;16</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>1</td>
<td>Series‡</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>&gt;16 (16–16)</td>
<td>4 (0.5–4)</td>
<td>0.2 (0.125–1)</td>
</tr>
</tbody>
</table>

In vitro susceptibility testing was performed according to the Clinical and Laboratory Standards Institute M38A2 method (32).

†Mutations that have previously been shown to be associated with azole resistance in *A. fumigatus* (7,20,21).

voriconazole and 0.5 mg/L for posaconazole) was observed in 12/84 (14.3%) and 44/84 (52.4%) of ITZ-positive isolates, respectively (Table 2).[20]

Sequence-based identification classified 82 ITZ-positive isolates as A. fumigatus and 2 as A. niger. Sequencing of the cyp51A gene showed a substitution of leucine for histidine at codon 98 in combination with a 34-bp tandem repeat in the gene promoter in 74 (90.2%) of 82 resistant A. fumigatus isolates (Table 2). Other cyp51A mutations were found in 3 isolates (substitutions G54W, P216L, and F219I) (Table 2). In 1 isolate, a series of mutations was found, and in the remaining 4 azole-resistant A. fumigatus isolates (4.9%), no mutations were found in the cyp51A gene.[8] Because A. niger has no known resistance mechanisms, the 2 azole-resistant A. niger isolates were not further analyzed.

**Prevalence of Azole Resistance.** A median of 100 isolates were screened each month, with a range of 78–140 isolates per month. The total number of screened clinical isolates per center ranged from 130 to 449 and were recovered from 84 to 238 patients. The collection of isolates over the study period and the distribution of the recovery of resistant isolates are shown in Figure 1. Overall, 82 (4.6%) of 1,792 screened A. fumigatus isolates were azole resistant. The per-patient analysis showed a prevalence of 5.3%. Figure 2 shows the prevalence of resistance in A. fumigatus per center. The prevalence of azole resistance in A. niger was 3.8%. No seasonal variation was observed.

![Figure 1](image-url) **Figure 1.** Number of screened Aspergillus spp. isolates per month (bars) and prevalence (%) of azole resistance (line), the Netherlands, 2007–2009. ITZ, itraconazole.
Patient Characteristics. The screened A. fumigatus isolates were cultured from 1,192 patients, while other Aspergillus spp. isolates were recovered from 193 patients. Among the patients who harbored A. fumigatus isolates, the predominant underlying diseases were cystic fibrosis (382/1,192 [32.1%]) and other pulmonary diseases (265/1,192 [22.2%]). Almost 12% (138/1,192) of the A. fumigatus isolates were from patients who had hematologic/oncologic diseases. The distribution of underlying diseases is shown in Table 3.

Characteristics of Patients with Azole-Resistant Isolates. The 82 confirmed ITZ-resistant A. fumigatus isolates were recovered from 63 patients. The patients’ ages ranged from 1 to 85 years, with a mean age of 48.9 years. The sex distribution was equal.

Overall, the distribution of underlying diseases in patients with an ITZ-resistant isolate was similar to that observed in the group with ITZ-susceptible isolates, with 1 exception. In the group of patients with ITZ-resistant isolates, significantly more patients had hematologic/oncologic diseases than in the group that harbored ITZ-susceptible isolates (13/63 [20.6%] and 125/1,129 [11.1%], respectively; p<0.05; Table 3).

Aspergillus disease caused by an ITZ-resistant isolate was diagnosed in 23 patients (36%). Invasive aspergillosis was diagnosed in 8 patients (13%), 6 with confirmed and 2 with probable cases (Table 4). Four of these patients were azole naive, and 1 patient had been previously treated with fluconazole, which has no activity against Aspergillus species. All A. fumigatus isolates from patients with azole-resistant invasive aspergillosis harbored the TR/L98H resistance mechanism (Table 4). All 5 patients who were treated with voriconazole monotherapy died within 3 months of receiving a positive culture result. For 3 patients, voriconazole therapy was switched to another class of antifungal compounds, i.e., echinocandin, polyene, or both, but only 1 of those 3 patients survived. Overall, 7 (87.5%) of 8 patients with azole-resistant aspergillosis died within 3 months (Table 4).

Noninvasive Aspergillus spp. disease with an ITZ-resistant isolate was diagnosed in 15 patients. Five patients had cystic fibrosis and allergic bronchopulmonary aspergillosis. Six patients had an aspergilloma; of these patients, 2 were immunocompromised, 1 because of AIDS and 1 because of Job syndrome. Three patients sought treatment for otomycosis; 1 patient had Aspergillus spp. sinusitis. Data on previous exposure to azoles were available for 14 patients with noninvasive Aspergillus spp. disease; and 9 (64.2%) of these patients were azole naive. The TR/L98H mutation was the dominant resistance mechanism, because it was present in 12 (80%) of 15 isolates. Overall, 14 (64.3%) of 22 patients with azole-resistant Aspergillus spp. disease and known status of azole exposure were azole naive at the time the resistant isolate was cultured.
Discussion
Azole resistance is widespread in clinical A. fumigatus isolates in the Netherlands, and resistant isolates were found at all participating university medical centers. The overall prevalence was 5.3%, which is in a similar range as observed in the fungus culture collection of the Radboud University Nijmegen Medical Center.[6] However, the prevalence varied widely between the centers, with 1 center showing azole resistance in 10 (9.5%) of 105 patients with a positive Aspergillus spp. culture. In a previous study, we reported a resistance prevalence of 12.8% among A. fumigatus isolates sent to our department by other hospitals in the Netherlands.[6] This higher resistance rate may have been due to a different selection of isolates. In this study, all Aspergillus spp. isolates cultured in the participating laboratories were screened for resistance, irrespective of their clinical relevance, while in our previous study the isolates that were sent to Nijmegen were from patients with Aspergillus spp. diseases for whom antifungal therapy was not successful.[6]

Two other studies have investigated the prevalence of azole resistance in Aspergillus spp. in the Netherlands. One study investigated a collection of 170 clinical A. fumigatus isolates recovered from 114 patients from 21 different medical centers in 1945–1998.[30] Three (1.8%) isolates were resistant to ITZ and were later found to harbor the TR/L98H resistance mechanism. These were the first TR/L98H isolates recovered in the Netherlands.[6] Another study investigated the prevalence of azole-resistant Aspergillus spp. isolates in the Netherlands by

Figure 2. Prevalence (%) of azole-resistant Aspergillus fumigatus infections in university medical centers, the Netherlands, 2007–2009.
Table 3. Underlying diseases of patients from whom azole-susceptible and -resistant *Aspergillus fumigatus* isolates were recovered, the Netherlands, 2007–2009

<table>
<thead>
<tr>
<th>Underlying condition</th>
<th>No. (%) patients with susceptible isolates, n = 1,129</th>
<th>No. (%) patients with resistant isolates, n = 63</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystic fibrosis</td>
<td>365 (32.3)</td>
<td>17 (27.0)</td>
<td>0.38</td>
</tr>
<tr>
<td>Pulmonary disease, excluding cystic fibrosis</td>
<td>251 (22.2)</td>
<td>14 (22.2)</td>
<td>1.00</td>
</tr>
<tr>
<td>Hematologic/oncologic disease</td>
<td>125 (11.1)</td>
<td>13 (20.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>Otorhinolaryngologic disease</td>
<td>63 (5.6)</td>
<td>3 (4.8)</td>
<td>0.78</td>
</tr>
<tr>
<td>Internal disease</td>
<td>85 (7.5)</td>
<td>8 (12.7)</td>
<td>0.14</td>
</tr>
<tr>
<td>Solid organ transplantation</td>
<td>26 (2.3)</td>
<td>2 (3.2)</td>
<td>0.66</td>
</tr>
<tr>
<td>Intensive care unit patient</td>
<td>31 (2.7)</td>
<td>2 (3.2)</td>
<td>0.84</td>
</tr>
<tr>
<td>Cardiac disease</td>
<td>9 (0.8)</td>
<td>1 (1.6)</td>
<td>0.50</td>
</tr>
<tr>
<td>Chronic granulomatous disease</td>
<td>5 (0.4)</td>
<td>0</td>
<td>0.60</td>
</tr>
<tr>
<td>Postoperative condition</td>
<td>23 (2.0)</td>
<td>1 (1.6)</td>
<td>0.81</td>
</tr>
<tr>
<td>Neurologic disease</td>
<td>2 (0.2)</td>
<td>0</td>
<td>0.74</td>
</tr>
<tr>
<td>Disease in children, not specified</td>
<td>4 (0.4)</td>
<td>0</td>
<td>0.64</td>
</tr>
<tr>
<td>Dermatologic disease</td>
<td>6 (0.5)</td>
<td>1 (1.6)</td>
<td>0.29</td>
</tr>
<tr>
<td>Other</td>
<td>91 (8.1)</td>
<td>1 (1.6)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>43 (3.8)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*x² test.
Table 4. Characteristics of patients with azole-resistant invasive aspergillosis, the Netherlands, 2007–2009

<table>
<thead>
<tr>
<th>Patient age, y/sex</th>
<th>Underlying disease</th>
<th>Disease</th>
<th>No. positive cultures†</th>
<th>Resistance mechanism</th>
<th>VCZ MIC, mg/L</th>
<th>Prior azole treatment (duration)‡</th>
<th>Treatment§</th>
<th>Outcome at 12 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>66/M</td>
<td>Lung carcinoma</td>
<td>Proven pulmonary aspergillosis</td>
<td>1</td>
<td>TR/L98H</td>
<td>4</td>
<td>None</td>
<td>VCZ</td>
<td>Died</td>
</tr>
<tr>
<td>59/M</td>
<td>Hematologic malignancy, allo-SCT, GvHD</td>
<td>Proven pulmonary aspergillosis</td>
<td>4</td>
<td>TR/L98H</td>
<td>8</td>
<td>VCZ (&gt;1 mo)</td>
<td>VCZ</td>
<td>Died</td>
</tr>
<tr>
<td>54/M</td>
<td>Acute myeloid leukemia, relapse, allo-HSCT</td>
<td>Proven pulmonary aspergillosis</td>
<td>1</td>
<td>TR/L98H</td>
<td>8</td>
<td>ITZ (2–4 wk)</td>
<td>VCZ</td>
<td>Died</td>
</tr>
<tr>
<td>50/M</td>
<td>Non-Hodgkin lymphoma, allo-SCT, GvHD, lung cavities</td>
<td>Probable pulmonary aspergillosis</td>
<td>2</td>
<td>TR/L98H</td>
<td>16</td>
<td>VCZ (&gt;1 mo)</td>
<td>VCZ</td>
<td>Died</td>
</tr>
<tr>
<td>36/F</td>
<td>Breast carcinoma with metastasis</td>
<td>Probable pulmonary aspergillosis</td>
<td>1</td>
<td>TR/L98H</td>
<td>1</td>
<td>None</td>
<td>VCZ</td>
<td>Died</td>
</tr>
<tr>
<td>13/F</td>
<td>Non-Hodgkin lymphoma</td>
<td>Proven pulmonary and CNS aspergillosis</td>
<td>1</td>
<td>TR/L98H</td>
<td>16</td>
<td>None</td>
<td>VCZ, CAS, AMB</td>
<td>Died</td>
</tr>
<tr>
<td>58/M</td>
<td>Liver transplantation for hepatic failure after methotrexate treatment for arteritis</td>
<td>Proven pulmonary and CNS aspergillosis</td>
<td>5</td>
<td>TR/L98H</td>
<td>2</td>
<td>None</td>
<td>AMB, VCZ</td>
<td>Died</td>
</tr>
<tr>
<td>60/M</td>
<td>Acute myeloid leukemia, allo-SCT, GvHD</td>
<td>Proven pulmonary and CNS aspergillosis</td>
<td>3</td>
<td>TR/L98H</td>
<td>4</td>
<td>FCZ (1–2 wk)</td>
<td>VCZ, CAS, AMB, POS</td>
<td>Survived</td>
</tr>
</tbody>
</table>

*VCZ, voriconazole; allo-SCT, allogeneic hematopoietic stem cell transplantation; GvHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; ITZ, itraconazole; CNS, central nervous system; CAS, caspofungin; AMB, amphotericin B; FCZ, fluconazole; POS, posaconazole.
† All cultures were Aspergillus fumigatus.
‡ Azole treatment <12 wk before the first culturing of an azole-resistant isolate.
§ Azole treatment after first culturing of resistant isolate.
using a collection of 209 unselected clinical *A. fumigatus* isolates obtained over a 6-month period in 2005.[31] Only 4 azole-resistant isolates were found, which corresponds with a prevalence of 1.9%.[31] The low prevalence may be because of the methods used. We used ITZ-containing agar-slants to select for resistant isolates, which enables detection of resistant colonies even in the presence of multiple azole-susceptible colonies. Although Klaassen et al.[31] did not explain how isolates were obtained, azole-resistant colonies could have been missed if regular culture media were used.[22,31] A $k$ coefficient of 0.81 indicates that screening for azole resistance by using agar supplemented with ITZ is a highly reproducible method and appears to be a feasible approach for screening for azole resistance and for surveillance studies.[34]

Most (74/82 [90.2%]) of the azole-resistant isolates in our study were found to possess the TR/L98H mutation in the *cyp51A*-gene, which is a similar proportion as reported previously.[6] Previous studies have suggested that the probability of 2 genomic changes developing during azole therapy is highly unlikely and that exposure of *A. fumigatus* to azoles in the environment may be a possible route of resistance development.[6,27,28] *A. fumigatus* isolates harboring the TR/L98H resistance mechanism were recovered from the environment and were genetically related to TR/L98H-containing clinical isolates.[28] This mode of resistance development implies that previous exposure of patients toazole compounds is not a prerequisite. Indeed, our study shows that 64% of patients with an azole-resistant isolate have no history of previous azole exposure during the 3 months before culturing the isolate. This finding supports the proposed environmental route of resistance development.

The prevalence and spread of TR/L98H in *A. fumigatus* in other countries are largely unknown. A recent study by Mortensen et al. showed that 8% of environmental *A. fumigatus* isolates in Denmark harbored the TR/L98H resistance mechanism, which indicates that TR/L98H may be spreading.[29] Because in vitro susceptibility testing of *A. fumigatus* is not routinely performed in most laboratories, international surveillance studies are warranted.

Most TR/L98H isolates were resistant to voriconazole (79%), but a broad range of MICs was observed that varied from 1 mg/L to 16 mg/L. Reasons for this variation remain unclear. TR/L98H isolates may have developed additional mutations that confer voriconazole resistance but that are not associated with the *cyp51A* gene. Another possibility is that the tandem repeat, which serves as a transcriptional enhancer, causes varying levels of up-regulation of the *cyp51A* gene. This up-regulation may result in differences in the voriconazole phenotype. More research is needed to gain insight into this phenomenon.

The outcome for patients with azole-resistant invasive aspergillosis was dismal, with a mortality rate of 88%. None of the patients who were treated with voriconazole monotherapy were alive at 3 months; of 3 patients whose treatment was switched to another class of antifungal drugs, only 1 survived. A critical issue for future study is whether azole resistance is a determinant of the poor
clinical outcome of patients with azole-resistant aspergillosis. A previously reported case series of patients with primarily noninvasive Aspergillus spp. disease indicated that azole resistance was associated with treatment failure.[7] However, establishing a relation between resistance and treatment outcome in invasive aspergillosis is complex because of the multiple factors that contribute to treatment outcome, including underlying disease, drug exposure, timing and accuracy of diagnosis, and timing of antifungal therapy. Furthermore, data are lacking on the effects of in vitro susceptibility patterns of Aspergillus spp. isolates on treatment outcomes. This relationship was recently described by using Aspergillus spp. isolates recovered from patients enrolled in the Transplant-Associated Infection Surveillance Network.[35] The 12-week mortality rate for patients with culture-positive confirmed or probable invasive aspergillosis was 53%.[35] For patients with azole-susceptible A. fumigatus aspergillosis treated with voriconazole, the proportion of deaths was 48% (J.W. Baddley, pers. comm.).

In a study by Herbrecht et al.[2], a subset of 51 modified-intent-to-treat patients with definite (43 patients) or probable (8 patients) invasive aspergillosis had a positive culture with A. fumigatus with a wild-type susceptibility. The all-cause proportion of deaths in this group at 12 weeks was 39% (P. Troke, pers. comm.). Both these analyses show a lower death rate than observed in our study, which indicates that azole resistance may be associated with a poor outcome. The median MIC of voriconazole in the 8 patients with azole-resistant invasive aspergillosis was 4 mg/L, which is resistant.[20] Effects of an elevated voriconazole MIC on treatment outcome were supported by preclinical experimental results, in which mice infected with an A. fumigatus isolate that had a voriconazole MIC of 2 mg/L required a 2-fold higher voriconazole dose than did mice infected with a wild-type isolate to achieve a comparable survival rate.[25]

The low survival rate of patients whose treatment was switched from azole therapy to nonazole therapy could be due to the delay of initiation of treatment with an effective alternative drug or to the presence of cerebral disease. Overall, the number of patients with azole-resistant aspergillosis in our study was low, and additional studies are needed to confirm the relation between azole resistance and treatment failure.

Because specimens from patients with invasive aspergillosis seldom produce positive cultures, our study underestimates the number of azole-resistant cases. The diagnosis of azole resistance in culture-negative cases is a challenge because current biomarkers, such as galactomannan and 1,3-β-D-glucan, do not enable species identification, let alone in vitro susceptibility testing. Molecular tools have been shown to be able to detect mutations associated with azole resistance directly in tissue[36] or in respiratory specimens[37] but are not yet suitable for use in routine patient care. Molecular tools need to be developed that enable the rapid detection of multiple mutations, although only known mutations can be detected.

Our study shows that azole resistance in clinical A. fumigatus isolates is
widespread in the Netherlands and that the survival rate of patients with azole-resistant invasive aspergillosis is low. The dominance of the TR/L98H resistance mechanism and the high proportion of resistant isolates recovered from azole-naive patients support an environmental route of resistance development. We believe that continued surveillance is required in the Netherlands, as well as routine in vitro susceptibility testing of *A. fumigatus* isolates obtained from patients with *Aspergillus* spp. disease. International surveillance programs are also warranted to estimate the size of the emerging problem of azole resistance. Furthermore, the first-line therapy of patients with invasive aspergillosis should be reconsidered, at least in those centers with a high prevalence of azole resistance.

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34. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977;33:159–74.


Drawing made by a 6-year old boy who was suffering from acute lymphoblastic leukemia and pulmonary zygomycosis, who underwent lobectomy.
Chapter 4:

Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles.

Abstract

Background. Azole resistance is an emerging problem in Aspergillus fumigatus and complicates the management of patients with Aspergillus-related diseases. Selection of azole resistance may occur through exposure to azole fungicides in the environment. In the Netherlands a surveillance network was used to investigate the epidemiology of resistance selection in A. fumigatus.

Methods. Clinical A. fumigatus isolates were screened for azole resistance in 8 university hospitals using azole agar dilution plates. Patient information was collected using an online questionnaire and azole-resistant A. fumigatus isolates were analyzed using gene sequencing, susceptibility testing, and genotyping. Air sampling was performed to investigate the presence of resistant isolates in hospitals and domiciles.

Results. Between December 2009 and January 2011, 1315 A. fumigatus isolates from 921 patients were screened. A new cyp51A-mediated resistance mechanism (TR46/Y121F/T289A) was observed in 21 azole-resistant isolates from 15 patients in 6 hospitals. TR46/Y121F/T289A isolates were highly resistant to voriconazole (minimum inhibitory concentration ≥16 mg/L). Eight patients presented with invasive aspergillosis due to TR46/Y121F/T289A, and treatment failed in all 5 patients receiving primary therapy with voriconazole. TR46/Y121F/T289A Aspergillus fumigatus was recovered from 6 of 10 sampled environmental sites.

Conclusions. We describe the emergence and geographical migration of a voriconazole highly resistant A. fumigatus that was associated with voriconazole treatment failure in patients with invasive aspergillosis. Recovery of TR46/Y121F/T289A from the environment suggests an environmental route of resistance selection. Exposure of A. fumigatus to azole fungicides may facilitate the emergence of new resistance mechanisms over time, thereby compromising the use of azoles in the management of Aspergillus-related diseases.

Introduction

The medical triazoles itraconazole, voriconazole, and posaconazole are the primary antifungal agents currently used in the management of infections caused by the saprophytic mold Aspergillus fumigatus. These triazoles are clinically licensed for the prevention and treatment of both noninvasive Aspergillus diseases as well as invasive aspergillosis.[1] Acquired resistance in A. fumigatus has long been perceived as a manageable problem as resistance development during azole therapy has been reported with only a very low frequency.[2-4] However, culture may underestimate the presence of resistance[5], and A. fumigatus isolates that harbor a resistance mechanism are commonly resistant to multiple triazoles.[6-9]

In the Netherlands, a second route of resistance development was suggested in which clinical A. fumigatus isolates may have become resistant through environmental exposure to 14α-demethylase inhibitors (DMIs).[6,10-12] DMIs inhibit fungal Cyp51A activity and are abundantly used for crop protection
Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles.

Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. Five DMIs, from the triazole class, showed in vitro activity against *A. fumigatus* and were shown to have a molecule structure that was highly similar to that of the clinically licensed triazoles.[11] The environmental mode of resistance development is of major importance as >90% of Dutch clinical azole-resistant isolates are believed to have originated through this mode of resistance development.[6,7,12] The first resistance mechanism that is believed to be of environmental origin consists of a substitution at codon 98 in the *cyp51A* gene in combination with a 34 base-pair tandem repeat in the gene promoter (TR<sub>34</sub>/L98H).

TR<sub>34</sub>/L98H first emerged in clinical *A. fumigatus* isolates from Dutch patients in 1998 and a national surveillance study indicated that this resistance mechanism is now endemic in Dutch hospitals. *Aspergillus*-related diseases due to TR<sub>34</sub>/L98H included noninvasive infections and invasive aspergillosis, and infections were found to occur both in azole-treated as well as in azole-naive patients.[6,7] TR<sub>34</sub>/L98H is increasingly reported in other European countries, and more recently also in China and India.[6,13-23] Molecular typing studies indicate that the fungicide-driven route of resistance development carries the risk of geographical migration of this resistance trait, similar to azole-resistant phytopathogenic fungi.[24]

In our current study, we investigated the emergence of a new azole resistance mechanism in *A. fumigatus*. We describe the epidemiology and clinical implications, and performed environmental sampling to determine if the new resistance mechanism was present in our environment.

**Methods**

**Surveillance Network.** Between May 2009 and January 2011, all *Aspergillus* isolates cultured from clinical samples that were processed in medical microbiology laboratories of the 8 university medical centers in the Netherlands were routinely screened for the presence of azole resistance, irrespective of the clinical relevance of the culture result. An online questionnaire was completed in 7 of 8 centers for every collected isolate. The questionnaire included questions about isolate characteristics (species identification and date of isolation) and patient characteristics (age, sex, and underlying disease). In one center the questionnaire was only completed for patients from whom a resistant isolate was recovered.

**Screening: 4-Well Azole-Agar Dilution Plates.** *Aspergillus* colonies that grew in primary cultures were subcultured on a specially developed 4-well azole-agar dilution (4D) plate.[25] All wells contained Roswell Park Memorial Institute 1640 agar, and 3 wells were each supplemented with 1 of the azoles: itraconazole (4 mg/L), voriconazole (1 mg/L), or posaconazole (0.5 mg/L). The fourth well contained no azole and served as growth control. The 4D plates were incubated at 37°C and growth was assessed after 48 hours. For every isolate that was able to grow on any of the azole-containing wells, the primary culture isolate was
sent to the Radboud University Nijmegen Medical Centre for further analysis. For those isolates that grew only on the control well, the Web-based questionnaire was completed, but the isolate was considered azole-susceptible and not further analyzed. At the screening sites, the *Aspergillus* isolates were identified to the species level by conventional methods, that is, the ability to grow at 48°C and macro- and microscopic culture morphology.

**Analysis of *A. fumigatus* Isolates.** All *A. fumigatus* isolates that grew on 1 or more azole-containing agar wells were investigated for their antifungal susceptibility to itraconazole, voriconazole, posaconazole, and the DMI tebuconazole using the Clinical and Laboratory Standards Institute M38-A2 broth microdilution reference method.[26] For resistant isolates that were confirmed to exhibit a non-wild-type phenotype, the full coding sequence of the *cyp51A* gene and promoter region was determined by polymerase chain reaction (PCR) amplification and sequencing to detect any mutations (reference *cyp51A* sequence: GenBank accession number AF338659).[27] Molecular identification was performed by sequencing the highly conserved β-tubulin and calmodulin gene, as described previously.[28]

Microsatellite genotyping was used to investigate genetic distances between the isolates by analysis of 6 microsatellites (STRAf 3A, 3B, 3C, 4A, 4B, and 4C), as described previously.[6] If multiple resistant isolates were obtained from 1 patient, only the first isolate was included. For every resistant isolate, 2 control isolates were selected that had been cultured between 1 month before and 1 month after the date of isolation of the resistant isolate. One control isolate harbored the TR34/L98H resistance mechanism, whereas the other exhibited a wild-type phenotype. From the microsatellite data, allele sharing distance matrices were generated and these matrices were used as input for the Neighbor program of the PHYLIP software package (Phylogeny Inference Package version 3.6, Department of Genome Sciences, University of Washington, Seattle) to produce the dendrogram.[29]

**Patient Characteristics.** The Web-based database was used to retrieve clinical information of the patients with a culture yielding *A. fumigatus*. For patients harboring an azole-resistant isolate, the following additional information was recorded: the presence of *Aspergillus* disease, azole exposure within 12 weeks preceding the culture of the resistant isolate, treatment, and outcome at 12 weeks. Invasive aspergillosis was classified according to the European Organization for Research and Treatment of Cancer and Mycoses Study Group (EORTC/MSG) consensus definitions.[30] Human experimentation guidelines from the Committee on Research Involving Human Subjects Arnhem–Nijmegen were followed in the conduct of this research.

**Environmental Sampling.** The presence of *A. fumigatus* resistant to medical triazoles in the environment was investigated through sampling at the Radboud University Nijmegen Medical Centre, the University Medical Centre Groningen, and 8 domiciles (6 in the Nijmegen area and 2 in the Groningen area). Indoor
Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles.

sites and 1 site in the direct outdoor proximity were sampled. Air samples were obtained using a Casella air sampler (Casella Measurement, catalog number E7627/Z-24, serial number 026510/026514, London, UK). Cultures of airborne viable fungi were performed on Sabouraud agar. For selection of azole-resistant fungi, Sabouraud agar supplemented with itraconazole (4 mg/L) or voriconazole (1 mg/L) were used. The volume of air that was sampled was 14 000 L (700 L/minute for 20 minutes) to detect azole-resistant spores. The plates were incubated at 37°C and inspected twice daily for 4 days.

Any colony that grew on the agar supplemented with azoles was subcultured on a Sabouraud agar slant and was identified as *A. fumigatus* using colony morphology and microscopic characteristics. *Aspergillus fumigatus* isolates were screened for the presence of an insertion in the promoter region of the *cyp51A* gene by previously described PCR primers and conditions.[24] Tandem repeats of different sizes could be identified on the basis of the size of the amplified PCR fragment. Isolates containing the TR34 were screened for the presence of the L98H substitution by using 2 PCR reactions: an L98- and an L98H-specific PCR (primers described elsewhere).[24] Azole-resistant isolates without TR34/L98H were selected for sequencing the *cyp51A* gene and promoter region as described above.[27]

## Results

**Emergence of the New Resistance Mechanism.** In January 2010, a clinical *A. fumigatus* isolate originating from Nijmegen grew on the well containing voriconazole, and not on those containing itraconazole or posaconazole. In vitro susceptibility testing showed no activity of voriconazole (minimum inhibitory concentration [MIC] >16 mg/L), and attenuated activity of itraconazole (MIC 2 mg/L) and posaconazole (MIC 0.5 mg/L) (Table 1). Sequence analysis of the *cyp51A* gene showed the presence of 2 mutations leading to substitutions Y121F and T289A. In addition, a 46 base-pair tandem repeat was found in the gene promoter (TR34/Y121F/T289A). A second isolate was cultured in January 2010 from a patient in Amsterdam that exhibited a similar voriconazole-resistant phenotype and identical TR34/Y121F/T289A resistance mechanism (Table 1).

The culture collection of the surveillance network was then investigated for isolates with a voriconazole MIC of ≥16 mg/L. Since May 2009, when screening of isolates using the 4D plates had begun, 5 of 33 azole-resistant isolates were identified with a voriconazole MIC of ≥16 mg/L. Sequence-based analyses of the *cyp51A* gene of these isolates identified a third isolate, harboring the TR46/Y121F/T289A resistance mechanism, that had been cultured on 31 December 2009 in Utrecht. This was considered to be the first clinical isolate from our surveillance network to harbor the TR46/Y121F/T289A resistance mechanism.

**Prevalence of the TR46/Y121F/T289A Resistance Mechanism.** From December 2009 to January 2011, 1315 *A. fumigatus* isolates from 921 patients were screened for resistance in 7 of 8 university centers. In one center, the total number of
Table 1. Clinical Characteristics of 15 Patients From Whom an *Aspergillus fumigatus* Isolate Was Cultured That Harbored the TR44/Y121F/T289A Resistance Mechanism

<table>
<thead>
<tr>
<th>Sex/Age</th>
<th>Month of Isolation/Site</th>
<th>City</th>
<th>MIC (mg/L)</th>
<th>Underlying Condition</th>
<th>Aspergillus Disease [30]</th>
<th>Previous Azole Exposure&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Treatment</th>
<th>Outcome at 12 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>F/11</td>
<td>Dec 2009/sputum</td>
<td>Utrecht</td>
<td>ITZ 4</td>
<td>Relapse ALL, HSCT, GVHD</td>
<td>Probable IA</td>
<td>None</td>
<td>VCZ, CAS</td>
<td>Persistent infection</td>
</tr>
<tr>
<td>M/70</td>
<td>Jan 2010/ear</td>
<td>Amsterdam</td>
<td>VCZ &gt;16</td>
<td>Chronic otitis externa, sinusitis, and paralysis of abducens nerve</td>
<td>IA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>None</td>
<td>L-AMB, AND</td>
<td>Persistent infection</td>
</tr>
<tr>
<td>F/51</td>
<td>Jan 2010/abdominal abscess</td>
<td>Nijmegen</td>
<td>POS 0.25</td>
<td>Kidney transplant</td>
<td>Proven IA</td>
<td>None</td>
<td>VCZ, POS</td>
<td>Died</td>
</tr>
<tr>
<td>M/9</td>
<td>Feb 2010/sputum</td>
<td>Amsterdam</td>
<td>ITZ 4</td>
<td>Cystic fibrosis</td>
<td>No IA</td>
<td>None</td>
<td>None</td>
<td>Alive</td>
</tr>
<tr>
<td>M/69</td>
<td>Feb 2010/sputum</td>
<td>Amsterdam</td>
<td>VCZ &gt;16</td>
<td>Lung carcinoma, radiation</td>
<td>No IA</td>
<td>None</td>
<td>None</td>
<td>Alive</td>
</tr>
<tr>
<td>M/54</td>
<td>Mar 2010/sputum</td>
<td>Groningen</td>
<td>POS &gt;16</td>
<td>Multiple myeloma, autologous HSCT, relapse</td>
<td>Probable IA</td>
<td>None</td>
<td>VCZ, L-AMB</td>
<td>Died</td>
</tr>
<tr>
<td>F/54</td>
<td>Mar 2010/sputum</td>
<td>Groningen</td>
<td>ITZ 0.5</td>
<td>Cystic fibrosis, bilateral lung transplant</td>
<td>Proven IA</td>
<td>VCZ</td>
<td>L-AMB</td>
<td>Alive</td>
</tr>
<tr>
<td>F/65</td>
<td>May 2010/biopsy</td>
<td>Amsterdam</td>
<td>ITZ 1</td>
<td>Chronic otitis after cholesteatoma surgery</td>
<td>Proven IA</td>
<td>None</td>
<td>Surgery, L-AMB</td>
<td>Alive</td>
</tr>
<tr>
<td>M/76</td>
<td>May 2010/sputum</td>
<td>Amsterdam</td>
<td>ITZ &gt;16</td>
<td>Lung fibrosis</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Alive</td>
</tr>
<tr>
<td>M/70</td>
<td>Jun 2010/sputum</td>
<td>Amsterdam</td>
<td>ITZ 0.25</td>
<td>High energetic trauma, ICU admission</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Died</td>
</tr>
<tr>
<td>M/59</td>
<td>Jul 2010/brain biopsy</td>
<td>Amsterdam</td>
<td>ITZ 1</td>
<td>ß-thalassemia and diabetes mellitus</td>
<td>Proven IA</td>
<td>None</td>
<td>VCZ, L-AMB, CAS</td>
<td>Died</td>
</tr>
<tr>
<td>F/21</td>
<td>Sep 2010/sputum</td>
<td>Nijmegen</td>
<td>ITZ 0.5</td>
<td>Cystic fibrosis</td>
<td>ABPA</td>
<td>VCZ</td>
<td>None</td>
<td>Alive</td>
</tr>
<tr>
<td>F/49</td>
<td>Oct 2010/sputum</td>
<td>Groningen</td>
<td>ITZ &gt;16</td>
<td>COPD, unilateral lung transplant</td>
<td>None</td>
<td>None</td>
<td>L-AMB, VCZ</td>
<td>Alive</td>
</tr>
<tr>
<td>F/64</td>
<td>Nov 2010/sputum</td>
<td>Leiden</td>
<td>ITZ &gt;16</td>
<td>COPD</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Alive</td>
</tr>
<tr>
<td>F/50</td>
<td>Jan 2011/sputum</td>
<td>Utrecht</td>
<td>ITZ &gt;16</td>
<td>NH B-cell lymphoma, allo-SCT</td>
<td>Probable IA</td>
<td>VCZ</td>
<td>VCZ</td>
<td>Died</td>
</tr>
</tbody>
</table>

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; ALL, acute lymphoblastic leukemia; AND, anidulafungin; CAS, caspofungin; COPD, chronic obstructive pulmonary disease; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplant; IA, invasive aspergillosis; ICU, intensive care unit; ITZ, itraconazole; L-AmB, liposomal amphotericin B; MIC, minimum inhibitory concentration; NH, non-Hodgkin; POS, posaconazole; SCT, stem cell transplant; VCZ, voriconazole.

<sup>a</sup>Aazole exposure within 12 weeks preceding the culture of the azole-resistant isolate.

<sup>b</sup>This patient could not be classified according to the European Organization for Research and Treatment of Cancer and Mycoses Study Group consensus definitions. The patient showed bone destruction of the skull on computed tomography scan and *Aspergillus fumigatus* was recovered repeatedly from the ear, without any other explanation.
Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles.

isolates screened was unknown, as this center did not complete the online questionnaire for susceptible isolates. The overall prevalence of azole resistance was 6.8% (63 of 921 patients). Forty-seven of 63 patients (74.6%) harbored the TR34/L98H resistance mechanism and 13 patients (20.6%) TR46/Y121F/T289A. No cyp51A mutations were found in azole-resistant *A. fumigatus* isolates from 3 patients (4.7%). The prevalence of TR46/Y121F/T289A was 1.4% (13 of 921 patients) in this 14-month period. Besides this, 2 isolates with TR46/Y121F/T289A and 2 isolates with TR34/L98H were recovered from the center that had not recorded the total number of screened isolates. Therefore, within 14 months (December 2009 to January 2011), TR46/Y121F/T289A was detected in 21 clinical *A. fumigatus* isolates obtained from 15 patients in 6 different university hospitals in the Netherlands (Table 1, Figure 1).

**Characterization of TR46/Y121F/T289A Isolates.** The 21 TR46/Y121F/T289A isolates were identified as *A. fumigatus* based on sequence analysis of the β-tubulin and calmodulin genes, and voriconazole showed no in vitro activity against any of the isolates (MIC ≥16 mg/L). The activity of itraconazole and posaconazole was attenuated in all isolates (Table 1). The DMI tebuconazole, which has been shown to exhibit activity against wild-type *A. fumigatus* isolates,[11] showed no in vitro activity against TR46/Y121F/T289A isolates. One isolate with TR46/Y121F/T289A contained 2 additional substitutions, M172I and G448S. Recombinant experiments confirmed the association between the TR46/Y121F/T289A resistance mechanism and the observed phenotype (data not shown).

Microsatellite genotyping showed that clinical TR46/Y121F/T289A isolates clustered together. TR46/Y121F/T289A and TR34/L98H were separated into different clades and apart from wild-type control isolates (Figure 2).

**Clinical Characteristics.** Among the 15 patients identified with a TR46/Y121F/T289A isolate, 8 were diagnosed with azole-resistant invasive aspergillosis (Table 1). Three of these patients were classified as having probable disease and 4 as proven. One patient could not be classified according to the EORTC/MSG consensus definitions.[30] This patient showed bone destruction of the skull on computed tomography scan and *A. fumigatus* was recovered repeatedly from the ear, without any other explanation. All patients with invasive aspergillosis due to TR46/Y121F/T289A were azole-naive, except 1 patient with probable and 1 patient with proven invasive aspergillosis. At 12 weeks after recovery of the TR46/Y121F/T289A isolate, 4 of 8 patients with invasive aspergillosis had died and 2 patients had a persisting infection. All patients who died had received primary therapy with voriconazole. In 4 patients, primary therapy was initiated with liposomal amphotericin B. In 3 of these patients, invasive aspergillosis was diagnosed, and all patients were alive at 12 weeks (Table 1).
Environmental Sampling. A total of 140 azole-resistant *A. fumigatus* colonies were identified, recovered from 21 locations at 9 different sites (outdoor and indoor). *Aspergillus fumigatus* colonies could not be recovered from 3 samples (entrance of 1 of the hospitals and 2 domiciles) due to abundant growth of zygomycetes. Analysis of the *cyp51A* gene and the promoter region showed that 126 (90%) isolates harbored TR$_{34}$/L98H, and 14 (10%) harbored the new TR$_{46}$/Y121F/T289A resistance mechanism. Both resistance mechanisms were found in the Nijmegen and Groningen areas (Figure 1). In 6 of 10 sampled sites, the TR$_{46}$/Y121F/T289A resistance mechanism was found, and TR$_{34}$/L98H was recovered from 9 of 10 sites (Table 2). The genotypes of 11 of the 14 environmental TR$_{46}$/Y121F/T289A grouped together with the clinical TR$_{46}$/Y121F/T289A isolates, irrespective of the geographic site of recovery, whereas susceptible control isolates and TR$_{34}$/L98H isolates generally clustered in different clades (Figure 2).

Discussion
We describe the emergence and migration of a new azole resistance mechanism in *A. fumigatus*, a major cause of fungal diseases in humans. The new TR$_{46}$/Y121F/T289A resistance mechanism conferred high resistance to voriconazole and was associated with treatment failure in patients with invasive aspergillosis. Isolates harboring TR$_{46}$/Y121F/T289A were recovered from the environment, indicating that selection through a fungicide-driven route could have taken place.

TR$_{34}$/L98H was the first resistance mechanism that has been recovered from both clinical specimens and from the environment in the Netherlands. This resistance mechanism first emerged in clinical *A. fumigatus* isolates in
Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles.

A Dutch survey performed between 2007 and 2009 showed that TR34/L98H was widespread and that the prevalence varied between 0.8% and 9.5%.[7] TR34/L98H was found in azole-resistant *A. fumigatus* isolates that were recovered from environmental sources, such as soil and compost.[10] A second resistance mechanism reported in the Netherlands was a 53 base-pair tandem repeat (TR53) without mutations in the *cyp51A* gene. TR53 was associated with a pan-azole-resistant phenotype and was reported to have caused *Aspergillus* osteomyelitis in a pediatric patient in 2006.[31] Although a TR53 isolate was recovered from the environment (Verweij and Melchers, unpublished observations), there is currently no evidence for migration of this resistance mechanism. Our current study describes TR46/Y121F/T289A as the third resistance mechanism that has emerged in clinical and environmental isolates.

Similar to TR34/L98H,[11] the fungicide-driven route of resistance development could have caused the emergence of TR46/Y121F/T289A. Both resistance mechanisms consist of a combination of genomic changes that include a tandem repeat.[15,32] The new resistance mechanism included 3 genomic changes, and it appears unlikely that these would have evolved during azole therapy in all individual cases. Until now, only point mutations have been described to have developed in azole-resistant *A. fumigatus* isolates through patient therapy.[14,27] Both TR34/L98H and TR46/Y121F/T289A were recovered from epidemiologically unrelated patients, most of whom were azole-naive, and both were recovered from the environment. Furthermore, genetic typing showed clustering of TR34/L98H and TR46/Y121F/T289A in separate clades apart from wild-type isolates.

The evolving epidemiology of TR34/L98H indicates that this resistance mechanism is not restricted to the Netherlands but is increasingly being observed in other European Union member states[6,13-19,22,23] and outside Europe.[20,21] Genotyping indicates that in Europe, TR34/L98H isolates...
Table 2. Recovery of Azole-Resistant *Aspergillus fumigatus* Isolates Through Environmental Air Sampling of 14 000 L per Location

<table>
<thead>
<tr>
<th>Site</th>
<th>City</th>
<th>Location</th>
<th>No. of Resistant Colonies</th>
<th>TR&lt;sub&gt;46&lt;/sub&gt; Y121F/T289A</th>
<th>TR&lt;sub&gt;3d&lt;/sub&gt; L98H</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Nijmegen</td>
<td>Hospital restaurant</td>
<td>3</td>
<td>7</td>
<td>3</td>
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<tr>
<td></td>
<td></td>
<td>Hospital pediatrics department</td>
<td>9</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Outside hospital</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Schaijk</td>
<td>Living room</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basement</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<tr>
<td></td>
<td></td>
<td>Back yard</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
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<td>Schaijk</td>
<td>Living room</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>Back yard</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Overasselt</td>
<td>Kitchen</td>
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<td>5</td>
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<td></td>
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<td>Back yard</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Schaijk</td>
<td>Living room</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basement</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Back yard</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>Berghem</td>
<td>Living room</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Schaijk</td>
<td>Hall</td>
<td>13</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balcony</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>Groningen</td>
<td>Hospital entrance&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Outside hospital</td>
<td>29</td>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td>9</td>
<td>Scharmer</td>
<td>Living room</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Back yard</td>
<td>6</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>Garmerwolde</td>
<td>Living room&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Back yard</td>
<td>12</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> For these sites the agar plates, supplemented with azoles, were rapidly overgrown with zygomycetes, thereby precluding the opportunity to select suspected azole-resistant *A. fumigatus* colonies.
represent offspring of a common ancestor[24] and could have developed locally, possibly in the Netherlands, and subsequently spread across countries through wind-dispersed conidia or ascospores. Given the rapid geographical migration of TR_{46}/Y121F/T289A in Dutch hospitals, it can be anticipated that this resistance mechanism will spread, similar to TR_{34}/L98H. Indeed, recently a lethal case of azole-resistant invasive aspergillosis due to TR_{46}/Y121F/T289A was reported in a patient from the neighboring country of Belgium.[33]

Resistance threatens the outcome of patients with Aspergillus-related diseases, especially those with azole-resistant invasive aspergillosis. Voriconazole, which is recommended for the primary therapy of invasive aspergillosis, was uniformly inactive against TR_{46}/Y121F/T289A isolates, and treatment failed in all patients with proven or probable invasive aspergillosis who had received primary therapy with voriconazole. As the activity of itraconazole and posaconazole was also reduced in the majority of the isolates, the azole class appears not to be a treatment option in patients with infection with A. fumigatus harboring TR_{46}/Y121F/T289A.

There are limited clinical data regarding alternative treatment options in azole-resistant invasive aspergillosis. In vitro and experimental studies indicated that the combination of voriconazole and anidulafungin was synergistic against azole-susceptible infection but that in voriconazole-resistant disease, synergism was lost.[34,35] There is concern that in infection with isolates where voriconazole shows no activity, such as the TR_{46}/Y121F/T289A strains, the efficacy of the combination will rely solely on that of anidulafungin, which is suboptimal. [35] In a murine model of disseminated aspergillosis, liposomal amphotericin B was shown to be effective against azole-resistant A. fumigatus with various resistance mechanisms, including TR_{46}/Y121F/T289A.[36] In our current study, patients who received primary therapy with liposomal amphotericin B appeared to respond better than those receiving voriconazole, although the number of patients was very limited and the underlying conditions diverse.

Systematic surveillance through a network of clinical microbiology laboratories proved to be a useful strategy to detect the emergence and spread of the new resistance mechanism in A. fumigatus. Given the observed spread of azole resistance across Europe we believe that international surveillance programs are warranted. We believe that our observations are very worrisome as they indicate that continued use of triazole DMIs in our environment with activity against A. fumigatus will not only help resistance traits to sustain in the environment, but will also cause new resistance mechanisms to emerge. The potential of geographical migration, as observed with TR_{34}/L98H, indicates that the fungicide-driven route of resistance selection will not remain a regional problem. Unless we are able to implement measures that prevent fungicide-driven resistance selection, the clinical use of azoles will become severely compromised. It is therefore important to understand the conditions that allow for selection of resistance in the environment and to investigate which preventive measures might be effective.
Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.
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29. Park SDE. Trypanotolerance in West African cattle and the population genetic


Drawing made by a 16-year old girl suffering from acute lymphoblastic leukemia and pulmonary and cutaneous azole-resistant invasive aspergillosis.
Chapter 5:

Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus* (SCARE-network).

Abstract
Azole resistance has emerged in *Aspergillus fumigatus* but the frequency of resistance is unknown in many countries. Therefore, for one year 3,788 clinical *Aspergillus* isolates were screened for resistance at 22 centres in 19 countries. The overall prevalence of azole resistance in patients with *A. fumigatus* species complex (sc) isolates was 3.2% and ranged from 0 to 26.1% per centre. Sixty azole-resistant *A. fumigatus* sc isolates were recovered from 46 patients from 11 countries. Forty-seven of 60 azole-resistant isolates were classified as *A. fumigatus* by molecular identification and 13 as *A. fumigatus* sibling species: *A. lentulus* and *Neosartorya* species. TR34/L98H was the most frequent azole resistance mechanism, and was found in isolates originating from Austria, Belgium, Denmark, France, Italy and the Netherlands. Of 46 patients with an azole-resistant isolate 28 had documented *Aspergillus* disease and azole resistance was found in 10 of 195 patients with invasive aspergillosis (5.1%).

Introduction
*Aspergillus fumigatus* is a saprophytic mould that finds its ecological niche in decaying organic matter.[1] Conidia of *A. fumigatus* are widely dispersed in the air and after inhalation by a susceptible host *Aspergillus* is able to cause a wide spectrum of diseases, varying from chronic localized to acute invasive disease. [2] Invasive aspergillosis (IA) occurs mainly in severely immunocompromised patients and may be associated with high mortality rates.[3,4] The most important class of antifungal drugs with activity against aspergilli are the triazoles including the clinically licensed drugs itraconazole, posaconazole and voriconazole. Voriconazole is the recommended first choice compound for treatment of IA.[4,5]

Reports indicate that azole resistance in *A. fumigatus* is an emerging clinical problem.[6,7] New sibling species of *A. fumigatus* have been reported to cause IA, and are generally less susceptible to azole compounds compared to *A. fumigatus*.[8-10] Acquired azole resistance has been found mainly in *A. fumigatus* following chronic azole therapy.[11-14] In azole-treated patients with aspergilloma multiple resistance mechanisms were found in individual *A. fumigatus* colonies, indicating that in this specific setting the fungus is capable of rapidly adapting to azole exposure.[14]

An alternative route of resistance selection appears through environmental exposure of *A. fumigatus* to azole fungicides. It is suggested that in *A. fumigatus* resistance mechanisms are selected in the environment, and that these mechanisms reduce the efficacy of the medical triazoles because of similarities between the molecular structure of azole fungicides and the medical triazoles.[15-19] Unlike the patient route, the fungicide-driven route of resistance selection carries the risk of geographical migration. In the Netherlands, the resistance mechanism from environmental origin, a combination of mutations
in the cyp51A gene (TR34/L98H) is highly dominant, contributing to over 90% of resistance mechanisms found in azole-resistant isolates.[6,20] Azole resistance has been observed in patients with no recent history of azole therapy and the mortality in patients with azole-resistant IA was 88%.[20]

As in vitro susceptibility testing of Aspergilli is currently not routinely performed in most clinical microbiology laboratories, the global prevalence of azole resistance is not well characterized. The aim of our study was to investigate the prevalence of azole resistance in clinical Aspergillus isolates. A multicenter international surveillance network was set up in 2009 (Surveillance Collaboration on Aspergillus Resistance in Europe, SCARE-network). This network involved 18 European and 4 non-European sites.

### Materials and methods

#### Surveillance network. A multicentre international surveillance network was established, including 22 centres from 19 countries: Melbourne (Australia), Innsbruck (Austria), Leuven (Belgium), Rio de Janeiro and Porto Alegre (Brazil), Copenhagen (Denmark), Paris and Grenoble (France), Munster (Germany), London and Manchester (Great-Britain), Athens (Greece), Milan (Italy), Nijmegen (the Netherlands), Oslo (Norway), Gdansk (Poland), St Petersburg (Russia), Madrid (Spain), Stockholm (Sweden), Lausanne (Switzerland), Ankara (Turkey) and Birmingham (USA). The centres were asked to screen for azole resistance for a period of 12 consecutive months.

#### Screening method. To detect azole-resistant A. fumigatus a phenotypic screening-method was developed using a four-well plate format. Three of the four wells contained RPMI-1640 supplemented with itraconazole (4 mg/l), voriconazole (1 mg/l) and posaconazole (0.5 mg/l), respectively. The fourth well contained RPMI-1640 with no azole supplement and served as growth control. [21] All clinical Aspergillus isolates that were found in primary culture were subcultured on the four-well azole-agar plate, irrespective of its clinical significance, with a maximum of up to four colonies per clinical sample. For each screened isolate, patient characteristics were registered via an online questionnaire including age, gender, underlying disease, risk factors for invasive fungal disease (haematopoietic stem cell transplantation, corticosteroid use and intensive care unit admission), antifungal use in the three months prior to culture and presence of Aspergillus disease. Patients with IA were classified according to the EORTC/MSG consensus definitions, using radiologic imaging and microbiological results.[22] Two centres were reference laboratories with no access to clinical data. Human experimentation guidelines from the Committee on Research Involving Human Subjects Arnhem–Nijmegen were followed in the conduct of this research.

The four-well azole-agar dilution plates were prepared at the Radboud University Medical Centre Nijmegen, Department of Medical Microbiology, the Netherlands, and distributed to the other centres for proficiency testing.
centre was provided with a protocol and a blinded set of five *A. fumigatus* isolates. Two were multi azole-resistant (MICs: itraconazole >16 mg/l, voriconazole 4 and 8 mg/l, and posaconazole 0.5 mg/l), and three were susceptible. The centres were blinded for the resistance profiles and were asked to determine the ability of the isolates to grow on the four-well azole-agar dilution plates.

**Phenotypic and genotypic analyses.** Species identification was performed by macroscopic and microscopic morphology at the participating centres. Azole-agar dilution plates were incubated at 37ºC and growth was assessed after 48 hours. For every *A. fumigatus* isolate that was able to grow on any of the azole-containing wells, the primary culture isolate was sent to the Radboud University Medical Centre Nijmegen, the Netherlands and Statens Serum Institute, Denmark for susceptibility testing according to the EUCAST broth microdilution reference method.[23] The *in vitro* activities of itraconazole, voriconazole, posaconazole, and theazole fungicide tebuconazole were determined.

For every isolate that was classified as azole-resistant molecular species identification and mutation analysis were performed. Molecular species identification was performed by sequencing parts of the β-tubulin gene, as described previously.[8,9] If molecular identification classified the isolate as *A. fumigatus*, the full coding sequence of both strands of the *cyp51A* gene were determined by PCR amplification. PCR products were separated on an agarose gel, purified, sequenced, and aligned to the reference bank (GenBank AF338659) to detect any mutations, as described previously.[24]

For every confirmed resistant isolate one susceptible isolate was assigned, as being the first susceptible isolate recovered after the resistant isolate. Susceptibility testing and molecular identification were also performed on the susceptible isolates, as described above.

**Statistical analyses.** $\chi^2$ tests and ANOVA were performed by using SPSS version 20.0 (IBM, Somers, NY, USA).

**Results**

**Proficiency testing.** Nineteen of the 22 centres completed the proficiency testing. All centres correctly identified the two resistant isolates using the azole-agar dilution plates. Six centres incorrectly assessed a susceptible isolate as being resistant due to growth in the azole-agar after 48 hours of incubation. This was the case for one susceptible isolate in 1/22 centres, two susceptible isolates in 4/22 centres and all three susceptible isolates in 1/22 centres. Sensitivity of the azole-agar dilution plates for detection of resistance was therefore calculated to be 100%, with no false-negative results, but with an estimated rate of 21% false-positive results to arise from this study design (12/57 tests of susceptible isolates).
Participation in screening for resistance. A total of 3,788 *Aspergillus* isolates were screened for the presence of azole resistance by use of the four-well plates between January 2009 and January 2011. All centres screened for one year, except for one centre which screened for a 8-month period. The number of isolates screened per centre varied between 16 and 587, with a median of 82 (Figure 1).

Patient information was available from 1,911 patients from 21 centres from 18 countries. In one centre 264 isolates were screened, but patient information was not collected due to the fact that this was a mycology reference laboratory.

![Figure 1](image_url)  
**Figure 1.** Study characteristics (number of isolates/patients screened) from 22 centers in 19 countries participating in a study of azole resistance in *Aspergillus fumigatus*. *Period screened was 8 months instead of 1 year in this center for unknown reason. †Total number of screened patients is unknown because this center is a reference laboratory that does not have access to patient characteristics.*
laboratory. Patient age ranged from 0 to 97 years (mean: 49.8 years) and 55% of patients were males. The number of *Aspergillus* isolates screened per patient ranged from 1 to 17 (median 1).

**Isolate characteristics.** The majority of isolates (2,941 of 3,788, 77.6%) were classified as *A. fumigatus* species complex (sc). Among the remaining 847 isolates, *A. flavus* and *A. niger* sc were most frequent (Table 1). Most specimens (81.6%) were respiratory specimens, including sputum, tracheo-bronchial aspirates or bronchoalveolar lavage fluid (39.1%, 29.5% and 13%, respectively). Only a minority of isolates (2.2%) were recovered from other body sites including pleural fluid, pus, ascites, peritoneal fluid, blood, urine and cerebrospinal fluid. Tissue samples from normally sterile sites were responsible for 3.4% of the isolates and were obtained from biopsies and at autopsy (Table 1). Forty-five per cent of positive cultures from ear-swabs were *A. niger* species complex isolates.

**Patient characteristics for patients with *A. fumigatus* sc isolates.** *A. fumigatus* sc isolates were recovered from 1,450 patients. The most common underlying disease was chronic lung disease (30.0%), followed by cystic fibrosis (22.1%) and haemato-oncological diseases (12.9%)(Table 1). A total of 204 patients (14.1%) had undergone haematopoietic stem cell transplantation or solid organ transplantation and 265 patients (18.3%) had been treated with corticosteroids within three months prior to culture of the isolate. Two hundred and twenty three of 1,450 patients (15.4%) had received antifungals within three months prior to, or at the time of the positive culture. The majority (132/223, 59.2%) of these received azole monotherapy, including itraconazole, voriconazole or posaconazole, and 22 (9.9%) received combination therapy with an azole and a polyene or an echinocandin. The remaining patients were previously treated with fluconazole (32 patients), a polyene (16 patients) or an echinocandin (11 patients). Five patients received combination-therapy of a polyene drug together with an echinocandin. Finally, in five patients the antifungal treatment regimen was not specified.

For 806 patients (55.6%) the clinical relevance of the cultured *A. fumigatus* species complex isolate was reported and in 453 patients there was no evidence for *Aspergillus* disease. In 353 patients *Aspergillus* disease was diagnosed. In the group of patients in whom the relevance of the *Aspergillus* isolate was reported (806 patients) 23.3% (n=188) were diagnosed with possible (36 patients), probable (108 patients) or proven IA (44 patients) and 19.6% (n=158) were diagnosed with non-invasive *Aspergillus* disease. In 24 patients the infection was not classified. In the group of 158 patients with non-invasive disease most had aspergilloma (50 patients) or allergic bronchopulmonary aspergillosis (ABPA, 46 patients). Twenty-four patients were diagnosed with other forms of non-invasive disease, being chronic pulmonary aspergillosis and bronchitis, this entity was not further specified due to logical reasons on the online questionnaire. One patient suffered from a combination of ABPA with chronic pulmonary aspergillosis (Figure 2).
**Table 1.** Isolate characteristics of 3,788 screened *Aspergillus* isolates from 1,911 patients and underlying diseases in patients with *A. section Fumigati* (1,450).

<table>
<thead>
<tr>
<th>Species</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. section Fumigati</em></td>
<td>2,941</td>
<td>77.6</td>
</tr>
<tr>
<td><em>A. section Nigri</em></td>
<td>315</td>
<td>8.3</td>
</tr>
<tr>
<td><em>A. section Flavi</em></td>
<td>211</td>
<td>5.6</td>
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<tr>
<td><em>A. section Terrei</em></td>
<td>105</td>
<td>2.8</td>
</tr>
<tr>
<td><em>A. section Nidulanti</em></td>
<td>53</td>
<td>1.4</td>
</tr>
<tr>
<td>Other <em>Aspergillus</em> species</td>
<td>163</td>
<td>4.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specimen</th>
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<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>1,482</td>
<td>39.1</td>
</tr>
<tr>
<td>Bronchial secretion/ tracheal aspirate</td>
<td>1,116</td>
<td>29.5</td>
</tr>
<tr>
<td>Bronchoalveolar lavage fluid</td>
<td>494</td>
<td>13.0</td>
</tr>
<tr>
<td>Ear swab</td>
<td>261</td>
<td>6.9</td>
</tr>
<tr>
<td>Non-sterile (skin, nail, hair, faeces, mouth-wash)</td>
<td>155</td>
<td>4.1</td>
</tr>
<tr>
<td>Specimen from normally sterile tissue</td>
<td>129</td>
<td>3.4</td>
</tr>
<tr>
<td>Specimen from normally sterile fluid</td>
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<td>2.2</td>
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<td>36</td>
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<tr>
<td>Unknown/other</td>
<td>32</td>
<td>0.8</td>
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<table>
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<tr>
<th>Underlying diseases in 1,450 patients with <em>A. section Fumigati</em> isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic lung disease (excl. cystic fibrosis)</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>General internal disease (incl. auto-immune disease, AIDS, chronic granulomatous disease)</td>
</tr>
</tbody>
</table>

**Analysis of azole-resistant *A. fumigatus* sc isolates.** One-hundred eighty-one *A. fumigatus* sc isolates were able to grow on at least one of the azole-containing agar-wells and were tested according to the EUCAST broth microdilution reference method.[23] For 60 of 181 isolates (32.1%) the resistant phenotype was confirmed *in vitro* (Table 2), therefore the number of false-positive results on the azole-containing agar-plates was 121 of 2,941 isolates (4.1%). The 60 azole-resistant isolates were matched with 60 control isolates from each site which were unable to grow on the azole-containing wells of the screening plate. The *in vitro* susceptibility of these isolates is shown in Table 2.
Sequencing of the β-tubulin-gene classified 47 azole-resistant isolates (78.3%) as *A. fumigatus*. The other 13 azole-resistant isolates were classified as *A. lentulus* (n=7), *Neosartorya pseudofisheri* (n=4) and *N. udagawae* (n=2). In the control-group all 60 isolates were classified as *A. fumigatus*.

Sequence analysis of the *cyp51A* gene of *A. fumigatus* showed the presence of TR34/L98H in 23 of the azole-resistant *A. fumigatus* isolates (48.9%). All TR34/L98H isolates were cultured from patients from European centres (Vienna, Leuven, Copenhagen, Paris, Milan and Nijmegen). In three isolates from the Netherlands a new resistance mechanism was found, which consisted of a 46 base-pair tandem repeat in the promoter region combined with two substitutions in the *cyp51A* gene (TR46/Y121F/T289A).[25] Although a significant proportion of azole-resistant isolates (13 of 47, 27.7%) were recovered from patients from the UK, none of them harboured the TR34/L98H resistance mechanism. Overall, in 14 isolates other mutations were found in the *cyp51A* gene. Nine isolates had substitutions in recognised hotspots at codon M220 and four at codon G54. One isolate from the UK contained a point mutation (L329V) not reported to be associated with azole resistance.[12] In four isolates a series of mutations was...
found. In seven azole-resistant *A. fumigatus* isolates no mutations were found in the cyp51A-gene, indicating the presence of another, yet unknown, resistance mechanism (Table 3).

The *in vitro* susceptibility of the azole-resistant *A. fumigatus* (47 isolates), the *A. fumigatus* sibling species (13 isolates) and the control isolates (60 isolates) is shown in Table 2. All control isolates were susceptible to itraconazole, voriconazole and posaconazole. The *in vitro* activity of the azole fungicide tebuconazole is also shown in Table 2. The medical triazoles were less active against the *A. fumigatus* sibling group compared to *A. fumigatus*. However, the *A. fumigatus* sibling group was significantly more susceptible to itraconazole and posaconazole, when compared to the azole-resistant *A. fumigatus* isolates (p<0.05)(Table 2). The azole fungicide tebuconazole was in general more active, although the difference was not statistically significant, against the azole-resistant *A. fumigatus* group compared with the sibling species (p=0.09)(Table 2).

**Table 2.** Susceptibility for 3 antifungal medical azoles and 1 azole fungicide of resistant isolates (60 isolates) and control group (60 isolates) after species identification.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Median MIC, mg/L</th>
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</thead>
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<tr>
<td></td>
<td>Itraconazole</td>
</tr>
<tr>
<td>Azole-resistant</td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em>, n = 47</td>
<td>&gt;8</td>
</tr>
<tr>
<td><em>A. fumigatus</em> sibling species,* n = 13</td>
<td>1</td>
</tr>
<tr>
<td><em>A. fumigatus</em> controls, n = 60</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Aspergillus lentulus, Neosartorya pseudofischeri, *N. udagawae.*

**Prevalence of azole resistance.** In total, 60 azole-resistant isolates were recovered from 46 patients. Six isolates were from the reference laboratories without access to clinical details. The overall prevalence of azole resistance amongst patients with *A. fumigatus* sc isolates was 3.2%, which ranged from 0.0% to 26.1% between the centres. Azole resistance was detected in 11 of 19 (57.9%) participating countries. Azole resistance was not found in the centres from Brazil, Greece, Norway, Poland, Russia, Switzerland, Turkey and the USA. The probability of detecting resistance corresponded with the number of isolates that were screened per centre. In 88.9% of the centres where no resistance was detected less than 50 isolates per centre were screened within the 12 month period. This contrasted with the centres where azole resistance was observed, as in only 7.7% of these centres less than 50 isolates per centre had been screened (p<0.01).
<table>
<thead>
<tr>
<th>Country</th>
<th>No. azole-resistant isolates, n = 47</th>
<th>TR$^{34}$/L98H or TR$^{46}$/Y121F/T289A mechanism (no. isolates)</th>
<th>Other mutations (no. isolates)</th>
<th>No. isolates without Cyp51A-mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>2</td>
<td>TR$^{34}$/L98H (2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Belgium</td>
<td>8</td>
<td>TR$^{34}$/L98H (7)</td>
<td>F46Y/M172G (1)</td>
<td>0</td>
</tr>
<tr>
<td>Denmark</td>
<td>6</td>
<td>TR$^{34}$/L98H (4)</td>
<td>0</td>
<td>2</td>
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<tr>
<td>France</td>
<td>4</td>
<td>TR$^{34}$/L98H (1)</td>
<td>G54W (1)</td>
<td>2</td>
</tr>
<tr>
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<td>5</td>
<td>TR$^{34}$/L98H (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>The Netherlands</td>
<td>7</td>
<td>TR$^{34}$/L98H (4), TR$^{46}$/Y121F/T289A (3)</td>
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<td>0</td>
</tr>
<tr>
<td>Spain</td>
<td>1</td>
<td>No isolates</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sweden</td>
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<td>No isolates</td>
<td>F46Y/M172G</td>
<td>0</td>
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<tr>
<td>United Kingdom</td>
<td>13</td>
<td>No isolates</td>
<td>P381R/D481E (1), L329V (1), M220K (1), L77V/L399I/D481E (1), M220I (3), M220R (1), G54R (1), G54E (1), G54W (1)</td>
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</tbody>
</table>

Resistant isolates, % 100 55.3 29.8 14.9

**Table 3.** Acquired resistance mechanisms from each country in cyp51A gene in 47 *Aspergillus fumigatus* isolates with an azole-resistant phenotype.
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex/age</th>
<th>Country</th>
<th>Underlying condition</th>
<th>Corticosteroid use</th>
<th>Date/specimen</th>
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</thead>
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</tr>
<tr>
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<tr>
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</tr>
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<td>UK</td>
<td>oncologic disease</td>
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<tr>
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<tr>
<td>8</td>
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<td>Netherlands</td>
<td>hematologic malignancy, HSCT</td>
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<td>2009 Aug, sputum</td>
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</tr>
</tbody>
</table>

*ABPA, allergic bronchopulmonary aspergillosis; IA, invasive aspergillosis; AMB, amphotericin B; BAL, bronchoalveolar lavage fluid; CPA, chronic pulmonary aspergillosis; HSCT, hematopoietic stem cell transplantation; ITZ, itraconazole; POS, posaconazole; VCZ, voriconazole.
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex/age</th>
<th>Country</th>
<th>Underlying condition</th>
<th>Corticosteroid use</th>
<th>Date/specimen</th>
<th>Species</th>
<th>Resistance mechanism</th>
<th>Aspergillus disease</th>
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<th>Out- come</th>
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<td>G54W, Q249H</td>
<td>CPA</td>
<td>POS</td>
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</tbody>
</table>

*ABPA, allergic bronchopulmonary aspergillosis; IA, invasive aspergillosis; AMB, amphotericin B; BAL, bronchoalveolar lavage fluid; CPA, chronic pulmonary aspergillosis; HSCT, hematopoietic stem cell transplantation; ITZ, itraconazole; POS, posaconazole; VCZ, voriconazole.
Acquired resistance in *A. fumigatus* was found at 11 (50.0%) of the participating centres, which were all European: Austria, Belgium, Denmark, France, Italy, the Netherlands, Spain, Sweden and United Kingdom. Almost half (48.9%) of the azole-resistant isolates from these European countries harboured the TR34/L98H resistance mechanism. The prevalence of resistance at the centres where TR34/L98H was found, ranged from 2.1% to 6.0%. The highest prevalence of azole resistance was seen in patients from Manchester and London, United Kingdom (26.1% and 18.2%) and Nijmegen, the Netherlands (6.0%). Manchester serves as the National Aspergillosis Centre and many patients with complex chronic *Aspergillus* diseases are referred to this centre.

In five countries (Australia, Germany, Spain, Sweden and United Kingdom) azole-resistant *A. fumigatus* sibling species were recovered. In three of these countries acquired resistance in *A. fumigatus* was also found. In Australia the prevalence of intrinsic resistance was the highest (5.1%), whereas acquired resistance was not found.

**Characteristics of patients harbouring azole-resistant isolates.** From the 46 patients with resistant isolates, the majority suffered from chronic lung disease (20/46), cystic fibrosis (14/46) or haemato-oncologic disease (6/46). The underlying conditions in the remaining six patients were general internal disease, otomycosis, heart-transplantation, kidney-transplantation and post-surgery intensive care stay, respectively (Table 4). Fourteen patients had been previously treated with corticosteroids and ten were HSCT or SOT recipients. Twenty-three patients (50%) had been previously treated with antifungals.

Eight patients had azole-resistant *A. fumigatus* sibling isolates, whilst 38 had a resistant *A. fumigatus* isolate. Of these 38 patients, 19 had an isolate which harboured a fungicide-driven resistance mechanism, i.e. TR34/L98H or TR46/Y121F/T289A. When comparing patients with isolates harbouring presumed fungicide-driven resistance mechanisms with “non-fungicide driven” resistance mechanisms, i.e. point mutations or non-Cyp51A-mediated mechanisms, there was a significant difference in azole exposure: 4 of 19 patients (21.1%) with an isolate with the fungicide-driven resistance mechanism had a history of azole therapy, compared to 16 of 19 patients (84.2%) with isolates with other or no cyp51A mutations (p = 0.001)(Figure 2).

Of the 195 cases with IA, azole resistance was documented in ten (5.1%) (three proven, one probable and six possible infections). Amongst the patients with resistant isolates there were 28 patients with documented *Aspergillus* disease. Eight patients with a resistant isolate suffered from chronic pulmonary aspergillosis, three patients had aspergilloma, four patients were diagnosed with ABPA, one patient had chronic *Aspergillus* bronchitis, and one patient had otomycosis. One patient was diagnosed with chronic pulmonary aspergillosis but was also treated for ABPA. Eighteen of 46 patients (39.1%) did not have signs or symptoms suggesting invasive or non-invasive disease (Table 4). Case-fatality rate in our patient cohort with invasive *Aspergillus* disease was 70%.
Discussion
Our culture-based surveillance study showed that azole resistance is emerging among clinical A. fumigatus sc isolates. This substantiates our concern that azole resistance is an emerging problem in A. fumigatus. Studies in the United Kingdom and the Netherlands described the emergence of azole resistance since 1998. [6,7,20] Resistance has been sporadically reported over recent years in Austria, Belgium, China, Denmark, Germany, France, India, Sweden, Spain and USA. [13,26-35] In this study, however, acquired resistance was detected in 11 of 17 European centres in 9 different countries, resulting in an overall prevalence of azole resistance of 3.2%, with TR34/L98H being the predominant mechanism of resistance (48.9%). Noticeably, 37% of the global market of antifungal pesticides is spent in Europe.[19]

Previously TR34/L98H was found to be highly dominant in the Netherlands and this resistance mechanism is believed to be selected in the environment. Molecular typing evidence is accumulating that the TR34/L98H resistance alleles are clustered within a single lineage of Dutch populations of A. fumigatus. Furthermore, TR34/L98H isolates recovered from other European countries also clustered with the Dutch clonal population.[36] The clonal emergence of TR34/L98H suggests a common ancestor. As the earliest isolates harbouring TR34/L98H were recovered in the Netherlands, this might imply that this mechanism originated in this country. However, TR34/L98H has now also been reported in Asia and the Middle East.[28,29,37] Further studies are needed to help understand the origin and geographical migration of azole resistance in the environment. On the other hand, resistance in A. fumigatus may have remained undetected in other areas as in vitro susceptibility testing was not routinely performed. We believe that our study may even have underestimated the true prevalence of resistance as centres that screened less than 50 isolates failed to find resistance. Furthermore, a recent study suggested an underestimation of the prevalence of resistance, as detection of resistance by nucleic acid amplification techniques in respiratory samples from patients with chronic lung diseases showed azole resistance mechanisms in 55.1% of respiratory specimens.[38]

The TR34/L98H was found to be the most predominant resistance mechanism, albeit less frequent than previously reported in the Netherlands. This could be due to the mix of cases and the possible lower burden of TR34/L98H in the environment in other countries. Furthermore, in contrast to other azole resistance mechanisms, TR34/L98H was observed more often in patients without previous exposure to azoles. There was a predilection of isolates which harboured the TR34/L98H mutation for patients with acute invasive diseases when compared to patients with aspergilloma and chronic pulmonary aspergillosis. These observations support the environmental route of selection of TR34/L98H. [6,13,14,18]

Through this surveillance network the new resistance mechanism TR46/Y121F/T289A was found in Dutch A. fumigatus isolates. TR46/Y121F/T289A has been found previously in clinical and environmental isolates originating from
the Netherlands, and is associated with high level resistance to voriconazole. TR$_{46}$/Y121F/T289A was found to comprise a new clonal population, different from TR$_{34}$/L98H, that has the propensity for rapid geographical migration.[25] Indeed, TR$_{46}$/Y121F/T289A has also been recently recovered in a fatal case of IA in Belgium, and most recently from the environment.[39,40] Although speculative, it is tempting to anticipate that unless the underlying mechanisms for resistance selection in the environment are better understood and appropriate measures for prevention of dissemination are implemented, new populations of azole-resistant *A. fumigatus* will continue to emerge and migrate, thereby further compromising the use of the clinically-licensed azoles in human medicine.

Azole-resistant IA was documented in 5.1% of cases of IA, which is not lower than the percentage of the prevalence of azole resistance among the *A. fumigatus* isolates (3.2%). This may indicate that resistance does not come with a significant fitness cost, and that azole resistant isolates are at least just as capable of causing IA as non-resistant wild type isolates. Preclinical studies have shown that isolates with a cyp51A-mediated resistance mechanism (including the TR$_{34}$/L98H), exhibit similar virulence when compared to wild type isolates. [11] For isolates harbouring a fungicide-driven resistance mechanism any fitness cost would threaten its survival in the environment in competition with wild type isolates. The persistence and geographical migration of TR$_{34}$/L98H and TR$_{46}$/Y121F/T289A also suggests that these resistant strains are not less fit than wild types.

Although the clinical implications of sibling species of *A. fumigatus* are at present less well understood, our study confirms that these species are generally less susceptible to azole antifungal drugs than *A. fumigatus*.

Our study shows that azole resistance is widespread in Europe and causes *Aspergillus* diseases in our patients. Centres that care for patients with *Aspergillus* diseases should perform surveillance in order to determine their local epidemiology and perform MIC testing of clinically relevant *A. fumigatus* isolates. Furthermore, azole resistance has become a public health problem which needs continued surveillance at the international level and research on the mechanisms that enable its selection in the environment.

**Conclusion and perspective**

This report of the emergence of resistance on a global scale has launched a new phase in the management of *Aspergillus* diseases. A rapid and convenient screening method for resistance is indispensable, because of the rising frequency of azole resistance in *Aspergillus* isolates worldwide and its association with a worsened clinical outcome. The global migration of TR$_{34}$/L98H and TR$_{46}$/Y121F/T289A indicates that fungicide-driven resistance mechanisms are not a regional problem. Unless we are able to implement measures that prevent the fungicide-driven route of resistance development, the clinical use of azoles will be severely compromised.
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Conflicts of interest
We declare that we have no conflicts of interest related with this study. JL received travel grants from Gilead and MSD and honorarium as a speaker from Pfizer. MA has received research grants, travel grants and honorarium as a speaker or advisor from Astellas, Gilead, MSD and Pfizer. AW received educational grants from Pfizer, Gilead and MSD. KL received research grants from Gilead, Pfizer, Merck, Sharp & Dohme and served on the speakers bureau of Pfizer, Merck, Sharp & Dohme. SK received research funds, travel grants and honoraria as a speaker or advisor from Gilead, MSD and Pfizer. ED received research grants, travel grants and honorarium as a speaker or advisor from Astellas, Gilead, MSD, Bio-Rad, Ferrer International, Schering and Innothera. PG received travel grants and honoraria as a speaker from Gilead, MSD and Pfizer. JB served as a consultant for Merck, Pfizer, Astellas and Mayne Pharma. CM received travel grants from Astellas, honorarium as a speaker from Pfizer and grant support from Pfizer. CK received honoraria from Gilead, MSD, Pfizer and Astellas. AU received a travel grant from Astellas and honoraria as a speaker from MSD and Pfizer. LN received travel grants from MSD and Pfizer. PV received research grants, has attended conferences, given lectures and participated in advisory boards or trials sponsored by various pharmaceutical companies.

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References


Chapter 6:

General discussion and prospects for the future.
Our surveillance studies show that azole resistance in *A. fumigatus* is widespread. Coming from a practice that azole resistance was not routinely investigated in clinical microbiology laboratories, the presence and frequency of azole resistance is clearly underreported. Presently, institutions are starting to investigate azole resistance in clinical isolates and stored culture collections, and their result do confirm the widespread presence of azole resistance in *A. fumigatus*. In addition to Europe, azole resistance has been reported in the Middle East, Asia, Australia and most recently North and South America.[1-23] The anticipated potential of global migration has become a reality. Continued surveillance is essential to determine resistance frequencies at local, national and international level in order to determine trends in resistance frequency, distribution of mutations and possibly the emergence of new mechanisms. This is well illustrated in chapter 4 where the emergence of the TR$_{34}$/Y121F/T289A resistance mechanism was efficiently tracked through the existing surveillance network. This mutation is now also found globally and there is recent evidence that TR$_{92}$ and TR$_{128}$ resistance mechanisms have also emerged.[14-23, W.J.G. Melchers, personal communication] There are however limitations associated with current surveillance studies as they are based on screening for azole resistance of *A. fumigatus* isolated grown from clinical samples. It is well documented that *Aspergillus* culture has a low sensitivity and a recent audit in our hospital indicated that only 35% of BAL-cultures were positive in patients with hematological malignancy and persistent febrile neutropenia who underwent bronchoalveolar lavage as part of a diagnostic work-up for pulmonary infection (P.E. Verweij, unpublished data). As a consequence culture-based surveillance studies only reflect a minority of the patients with *Aspergillus* disease. Non-culture based assays such as PCR could be used to detect azole resistance mutations directly in clinical specimens. This has been shown to be feasible using in-house PCR. [24] Recently a commercial PCR became available (AsperGenius, PathoNostocs, Maastricht), which enabled the detection of two frequent mutations (TR$_{34}$/L98H and TR$_{46}$/Y121F/T289A).[25] Initial studies indicate that this allows detection of the mutations in culture-negative BAL samples. In a study from Denning and colleagues in 2011, azole-resistant *Aspergillus* DNA was found in 55.1% of culture-negative BAL-samples.[26,27] Such an approach could also be used for surveillance and allow more exact estimates of azole resistance than through culture surveillance only. However, the detection of a low load of *Aspergillus* DNA in plasma and serum remains problematic, and more sensitive non-culture based assays for these specimen still have to be developed.

Although our surveillance studies have shown that the incidence of azole-resistance among *A. fumigatus* vary considerably between institutes, recent studies have shown that even within a single institute, incidences may vary between departments. In one study, an incidence of 26% was found in culture-positive ICU-patients, which was much higher than the incidence in other hospital departments (14%; p=0.06). An explanation for the intra-hospital
differences of incidences may be found in the fact that treatment of patients with azole-resistant disease fails necessitating transferal to the ICU due progressive fungal disease.[28] In another study the primary A. fumigatus cultures were investigated from hematology and ICU-patients. A higher incidence of azole-resistance was found in hematology patients compared with ICU (26% versus 4.5%, respectively).[29] These observations are of importance for clinical decision making regarding the choice of primary therapy in patients suspected of invasive aspergillosis. An international expert panel recently recommended to consider avoiding azole monotherapy as initial antifungal therapy when the incidence of azole-resistance exceeded 10%.[30] This would indicate that detailed audits are required to establish the local resistance frequencies in different patient groups.

‘Environmental’ resistance mechanisms were found to be the dominant cause of azole-resistant Aspergillus disease in our surveillance studies. The clinical implications of this route of resistance are considerable as we were unable to identify risk factors for azole-resistant Aspergillus disease. Between 64% and 71% of patients had no history of previous azole therapy. Furthermore, unlike resistance mediated through patient therapy, any Aspergillus disease in any patient could be caused by environmental resistance mutations. Clinical microbiology laboratories need to perform in vitro susceptibility testing of clinical A. fumigatus isolates to determine their local azole-resistance incidence. In addition, A. fumigatus isolates recovered from clinical samples need to be subjected to susceptibility testing if antifungal treatment is required. If MIC-testing of Aspergilli is not available locally, isolates should be sent to a mycology reference laboratory. However, diagnosing azole-resistant Aspergillus disease is difficult even in culture-positive patients, as cultures may harbor azole-susceptible and azole-resistant colonies concomitantly.[31] It will be difficult for clinical microbiology laboratories to determine which colonies are to be selected for in vitro susceptibility testing. The approach of sub-culturing A. fumigatus colonies on agar supplemented with azoles, which was used in our surveillance studies is attractive for patient management as well.[32] Colonies that grow on the azole-supplemented agar have a high probability of resistance and these could be selected for MIC-testing or sent to a mycology reference laboratory. Initiatives to commercialize the agar-based screening agar (VIPcheck™) are taken in order to make this approach available to clinical microbiology laboratories. Growth on azole-containing agar may prompt the treating physician to move away from azole monotherapy, while awaiting MIC results, as the likelihood of resistance in those isolates is very high.

Since 1998 three resistance mechanisms have been found in both environment and patients in the Netherlands: TR34/L98H since 1998; TR53 since 2006 and TR92/Y121F/T289A since 2009. The emergence of new mutations over time indicates that the current use of azole fungicides is not durable, and new mutations will continue to emerge. Indeed very recently a fourth resistance mechanism (TR92/Y121F/M172V/T289A/G448S) was found in the environment

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and in clinical isolates (P.E. Verweij, unpublished data). As in medicine we are confronted with the consequences of azole resistance, every effort should be made to prevent new mutations emerging in the environment. Azole fungicides have an important role in food production and prevention of spoilage, and therefore any measures should be aimed at retaining the use of azoles both in medicine and for food production. Effective measures can only been designed if resistance selection in the environment is understood. Research should be aimed at understanding how and when azole resistance mutations arise in *A. fumigatus* in order to determine which interventions will prevent this occurring. Some investigators argue that the application of azoles is important for resistance selection, rather than the volume that is used.[33,34] Exposure to high concentrations may comprise the greatest risk, although this is debated by others.[35] Nevertheless sites which are considered at high risk for resistance should be investigated as a first step to unravel resistance selection. Such research requires international and multidisciplinary collaboration, bringing together researchers from different fields including mycology, genetics, epidemiology, medicine and agriculture. However, the urgency of the resistance problem needs to be recognized by governments and scientific communities. Although a sense of urgency regarding bacterial resistance is apparent at this level, funds to investigate fungal resistance should also be made available. Moreover, it is likely that both fields may benefit from each other when addressing the emerging threats in parallel.

In the meantime, the number of patients and physicians confronted with azole resistance will continue to increase. The high mortality rates that we have observed in our surveillance studies have been confirmed by others. [36,37] The presence of azole resistance probably at least doubles the mortality rate in patients with invasive aspergillosis. As we have included only culture-positive patients in our studies, it is likely that their disease is more progressed (compared with culture-negative patients) and therefore mortality rates should be compared with those of patients with culture-positive azole-susceptible invasive aspergillosis. To obtain a more accurate estimate of the attributable mortality of azole resistant invasive aspergillosis patients should be matched with controls with disease caused by azole-susceptible isolates. Given the fact that azole resistant invasive aspergillosis is still uncommon, a prospective multicenter registry enrolling patients with azole-resistant and –susceptible invasive aspergillosis would be an appropriate study design.

The current treatment guidelines do not incorporate azole resistance, although an ESCMID-guideline on the management of diseases caused by azole-resistant *A. fumigatus* is due shortly. An expert panel recently discussed the management of azole resistance providing care-pathways to aid physicians to make clinical decisions.[30] In the absence of conclusive clinical studies, the experts relied on their own experience and preclinical studies. Most experts favored liposomal amphotericin B for the treatment of patients with documented
azole-resistant *Aspergillus* diseases. However, opinions differed when addressing at which threshold azole monotherapy should be avoided. Most experts recommended a threshold of azole prevalence of 10% and alternative primary treatment options included voriconazole in combination with an echinocandin or liposomal amphotericin B. Some experts regarded liposomal amphotericin B less effective than voriconazole, but a head-to-head comparison of the two drugs has never been performed. Clearly more research is needed to determine the best approach in regions with high resistance rates. Other combinations, such as liposomal amphotericin B in combination with voriconazole, may be attractive as empirical therapy but requires further studies. However, some manifestations of azole-resistant *Aspergillus* disease have no alternative treatment option with comparable efficacy to a mould-active azole. The most pressing example is CNS aspergillosis that is virtually untreatable when the use of voriconazole is precluded. The mortality rate of azole-resistant CNS aspergillosis will approach that of the pre-azole era, e.g. 95% to 99%. New antifungal drug targets are needed to improve the prognosis of these patients although this will take many years before such drugs are clinically available. Therefore the efficacy of ‘old’ drugs, such as flucytosine and rifampicine, or alternative administration routes, i.e. directly into the CSF will need to be explored.
References


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Appendix:

Summary and main conclusions
Samenvatting en conclusies
Dankwoord
Curriculum Vitae
List of publications
Oral presentations at (inter)national conferences and symposia
Summary and main conclusions

*Aspergillus* is a saprophytic mould, which is, due to its sporulating capacity, spread worldwide through the environment. As a consequence of new tools of species identification, i.e. multilocus sequence-based phylogenetic analyses, numerous *Aspergillus* species have been identified to be able to cause a wide variety of diseases. In this thesis, we focused on disease caused by *Aspergillus fumigatus* (*A. fumigatus*) being the main cause of acute (angio-)invasive and chronic pulmonary aspergillosis. For adequate treatment of patients with aspergillosis, besides a prompt diagnosis, prompt initiation and targeted antifungal treatment is of utmost importance. Currently there are 3 classes of antifungal drugs with activity to *A. fumigatus*, being the polyenes, azoles and echinocandins. Besides the fact that the recent taxonomic changes have led to the classification of cryptic species of *A. fumigatus* being intrinsically less susceptible to the azole-class of antifungals, acquired azole-resistance in *A. fumigatus* sensu stricto is emerging.

The studies as presented in this thesis were performed to obtain a better insight in the frequency of azole resistance through surveillance of clinical *A. fumigatus* isolates. In addition to the investigation and description of the epidemiology of azole resistance, its distribution and trends in resistance mechanisms, we also aimed to identify risk factors associated with azole-resistant *Aspergillus* disease.

**Chapter 2** describes the characteristics of azole resistance selection through patient therapy. Nine consecutive isogenic *A. fumigatus* isolates cultured from a patient with pulmonary aspergilloma and azole therapy were investigated for azole resistance. The first cultured isolate showed a wild-type phenotype, but four azole-resistant phenotypes were observed in the subsequent eight isolates. Using a Cyp51A homology model and recombination experiments, in which mutations were introduced into a susceptible isolate, two substitutions at codons P216 and F219 were shown to be both associated with resistance to itraconazole and posaconazole for the first time, besides a mutation at codon G54, which previously showed to be associated with resistance. We performed a review of the literature and showed that in patients who develop azole resistance during therapy, multiple resistance mechanisms commonly emerge. The patients were probably initially colonized or infected with a susceptible isolate, and over time, through azole exposure, resistance developed in this initial isolate. In our review all patients were diagnosed with aspergilloma and chronic pulmonary aspergillosis. It was previously suggested that in the case of aspergilloma or cavitary aspergillus disease, the fungus is able to undergo multiple generations in the patient by the asexual way of reproduction. Sporulation (as can be observed in cavitary *Aspergillus* lung disease) as opposed to invasive hyphal growth may be important to facilitate the expression of the azole-resistant phenotypes. The median time between the last cultured wild-type isolate and the first azole-
resistant isolate was 4 months (range 3 weeks to 23 months), indicating a rapid induction of resistance.

In Chapter 3, the results of a prospective nationwide multicenter surveillance study to obtain insight in the extensiveness and consequences azole resistance on patient management strategies and public health, is presented. All Aspergillus isolates cultured from clinical samples from 7 university medical microbiology laboratories in the Netherlands were routinely subcultured on Sabouraud agar slants supplemented with 4 mg/L of itraconazole. Isolates able to grow on those slants were sent in to our laboratory for genotypic and susceptibility analyses. Clinical data was obtained as well. In total, 2,062 isolates from 1,385 patients were screened and the prevalence of itraconazole resistance in A. fumigatus was 5.3%. Patients with a hematologic or oncologic disorder were more likely to harbor an azole-resistant isolate than were other patient groups (p<0.05). Most patients (64%) from whom a resistant isolate was identified were azole naïve and the majority (90%) harbored the same resistance mechanism (TR34/L98H), indicating an environmental route of resistance development. The case-fatality rate of patients with azole-resistant invasive aspergillosis (8 patients) was 88%. In summary, the results of this nationwide study showed that multi-azole resistance in A. fumigatus is widespread in the Netherlands with a very high mortality rate among patients with invasive aspergillosis caused by an azole-resistant A. fumigatus isolate.

Chapter 4 underscores the necessity of continuation of surveillance for detection of azole-resistance, as a new resistance-mechanism was detected after continuation of the previously described surveillance in the Netherlands. A new cyp51A-mediated resistance mechanism (TR46/Y121F/T289A) was observed in 21 azole-resistant A. fumigatus isolates from 15 patients in 6 hospitals by using a further developed screening method, using 4-well Sabouraud agar plates supplemented with three azoles (itraconazole, voriconazole and posaconazole). TR46/Y121F/T289A isolates were highly resistant to voriconazole. Eight patients presented with invasive aspergillosis due to TR46/Y121F/T289A, and treatment failed in all 5 patients receiving primary therapy with voriconazole. Thereby, TR46/Y121F/T289A A. fumigatus is severely compromising our first-line treatment of invasive aspergillosis with voriconazole. In order to examine the prevalence of this new resistance mechanism in A. fumigatus we performed an environmental screening by obtaining air samples from several Dutch geographically unrelated areas. TR46/Y121F/T289A A. fumigatus was recovered from 6 of the 10 environmental sites and were genetically related to clinical resistant isolates, emphasizing the environmental route of resistance development.

As azole resistance in A. fumigatus was emerging in the Netherlands, there was a growing need for examination of the global burden of this problem. Therefore we set up an international screening program for detection of azole resistance worldwide. In Chapter 5, the results of an international screening program performed within the context of a prospective multicenter international
surveillance study are presented. A total of 3,788 *Aspergillus* isolates were screened in 22 centers from 19 countries, by using the Dutch 4-well plates. Azole-resistant *A. fumigatus* was more frequently and more widespread found than previously acknowledged, and was detected in 11 of 17 participating European centers in 9 countries. An overall prevalence of azole resistance worldwide was calculated at 3.2%. A majority of isolates from patients with acute invasive aspergillosis harbored the TR34/L98H mutation, but was not observed in patients with aspergilloma and chronic pulmonary aspergillosis. Azole-resistant invasive aspergillosis was documented in 5.1% of patients of invasive aspergillosis and was associated with a dismal outcome. To conclude, azole-resistant *A. fumigatus* is not restricted to the Netherlands, but is encountered on a global scale. The management of disease caused by azole-resistant *A. fumigatus* should be reconsidered within the lights of these results.
Samenvatting en conclusies

Aspergillus is een saprofytaire schimmel, welke zich door zijn sporenvormende eigenschappen wereldwijd door de omgeving heeft verspreid. Ten gevolge van nieuwe methoden om species te identificeren, zoals multilocus sequence-based fylogenetische analyses, zijn inmiddels een groot aantal Aspergillus species geïdentificeerd als veroorzakers van een breed scala aan ziektebeelden. In dit proefschrift werd voornamelijk gericht op Aspergillus fumigatus (A. fumigatus), welke acute (angio-)invasieve en chronische pulmonale aspergillose kan veroorzaken. Voor een adequate behandeling van patiënten met aspergillose zijn, behoudens een zo vroeg mogelijke diagnose en tijdige start van behandeling ook de doelgerichtheid ervan cruciaal. Op dit moment zijn 3 drie verschillende klassen antifungale middelen beschikbaar die gebruikt kunnen worden tegen A. fumigatus, zijnde polyenen, azolen en echinocandines. Naast het feit dat alle recent taxonomische ontwikkelingen hebben geleid tot de ontdekking van cryptic species die intrinsiek resistent zijn voor azolen, is ook verworven resistentie bij A. fumigatus sensu stricto een opkomend probleem.

De onderzoeken beschreven in dit proefschrift zijn verricht om meer inzicht te verkrijgen in het vóórkomen van azool-resistentie bij klinische A. fumigatus isolaten doormiddel van surveillance programma’s. Naast de beschrijving van de epidemiologie van azool-resistentie, door onderzoek naar de verspreiding ervan en trends in resistentie mechanismen, hebben we gemeend om risicofactoren voor het verkrijgen van azool-resistente Aspergillus ziekte te identificeren.

In Hoofdstuk 2 wordt ingegaan op de karakteristieken van het verkrijgen van azool-resistentie ten gevolge van de behandeling van patiënten met azolen. Negen isogenetische A. fumigatus isolaten werden achtereenvolgens gekweekt uit een patiënt met een pulmonaal aspergilloom en azool-behandeling. Alle isolaten werden onderzocht op de aanwezigheid van azool-resistentie. Het eerste isolaat was een wild-type isolaat, maar 4 verschillende resiente fenotypen werden ontdekt in de 8 daaropvolgende isolaten. Met behulp van een Cyp51A homologie-model en recombinant analyses werden 2 mutaties (substituties in codon P216 en F219) ingebracht in een wild-type azool-gevoelig isolaat, waarbij beide mutaties geassocieerd konden worden met resistentie voor itraconazol en posaconazol. Tevens werd een mutatie gevonden in codon G54, welke al eerder werd gerelateerd aan azool-resistentie. Daarnaast hebben we een literatuuronderzoek verricht, waarbij we lieten zien dat in patiënten die behandeld worden met azolen vaak een diversiteit aan mutaties wordt gevonden. Waarschijnlijk zijn deze patiënten initieel gekoloniseerd of primair geïnfecteerd met een gevoelig isolaat, welke door blootstelling aan azolen in de loop van de tijd resistentie heeft ontwikkeld. In dit gepresenteerde literatuuroverzicht leden ook alle patiënten aan chronische pulmonale aspergillose of hadden een aspergilloom, hetgeen overeenkomt met eerdere reports waarin wordt gesuggereerd dat in het geval van een aspergilloom of bij
holtevormende aspergillusziekte, de schimmel in de mogelijkheid wordt gesteld om zich middels asexuele reproductie te kunnen voortplanten en zo meerdere generaties te kunnen voortbrengen. Sporulatie, in plaats van doorgroei van hyfen, bij deze specifieke ziektebeelden zou kunnen zorgen voor expressie van de verschillende resistente fenotypen. Overigens is de mediane tijd tussen de laatst gekweekte wild-type isolaat en eerste resistente isolaat gemiddeld 4 maanden (met een range van 3 weken tot 23 maanden), wat een snelle inductie van resistentie betekent.

In **Hoofdstuk 3** wordt een prospectieve, nationale, multicenter surveillance beschreven, welke werd opgezet om meer inzicht te verkrijgen in de uitgebreidheid en gevolgen van azool-resistentie voor patiënt-management strategieën en public health. Hiervoor werden alle gekweekte *Aspergillus* isolaten uit klinische materialen van 7 Nederlandse universitaire medisch-microbiologische laboratoria routinematig overgekweekt in reageerbuizen met een Sabouraud agar-medium waaraan 4 mg/L itraconazol was toegevoegd. Alle isolaten welke groeiden in deze buizen werden ingestuurd naar ons laboratorium van het Radboud UMC Nijmegen, tezamen met klinische data van de patiënt, ten behoeve van statistische, genotypische en gevoeligheidsanalyses. In totaal werden 2.062 isolaten van 1.385 patiënten gescreend op resistentie voor itraconazol, en werd een prevalentiecijfer berekend van 5,3% (range 0,8%-9,5%) onder patiënten, waarbij patiënten met hematologisch of oncologisch onderliggend lijden eerder een azool-resistent isolaat leken te verkrijgen dan patiënten met een ander onderliggend lijden (p<0,05). De meeste patiënten (64%) waaruit een resistent isolaat werd gekweekt waren nooit voorbehandeld met azolen en de meerderheid (90%) van de isolaten vertoonden hetzelfde resistentie mechanisme (TR 34L98H), hetgeen aangeeft dat blootstelling van *A. fumigatus* aan azolen in de omgeving een andere plausibele oorzaak is voor ontwikkeling van resistentie. De mortaliteit onder patienten met azool-resistente invasieve aspergilllose (8 patiënten) bedroeg 88%. Samengevat, toonde deze nationale studie aan dat multiazool-resistentie bij *A. fumigatus* wijdverspreid voorkomt in Nederland en dat het geassocieerd met een hoge mortaliteit onder patienten met invasieve azool-resistente aspergilllose.

**Hoofdstuk 4** ondersteunt het belang van voortgaande surveillance voor de detectie van azool-resistente isolaten, daar een nieuw resistentie-mechanisme werd ontdekt na continuerende van de eerder beschreven surveillance in Nederland. Een nieuw cyp51A-gemedieerd resistentie-mechanisme (TR 46/Y121F/T289A) werd gezien bij 21 azool-resistente isolaten van 15 verschillende patiënten uit 6 ziekenhuizen, middels een verder ontwikkelde screeningsmethode met 4-wells Sabouraud agar platen welke 3 verschillende azolen bevatten (itraconazol, voriconazol en posaconazol). TR 46/Y121F/T289A isolaten bleken resistant voor voriconazol. Acht patiënten leden aan invasieve aspergilllose met TR 46/Y121F/T289A, en de behandeling faalde bij alle 5 de patiënten die voriconazol ontvingen als eerste-keus therapie. Daarom zorgt TR 46/Y121F/T289A *A. fumigatus*
voor een ernstige tekortkoming van onze eerste-keus behandeling van invasieve aspergillose met voriconazol. Daarnaast werden luchtmonsters genomen op diverse geografisch-ongerelateerde regio’s in Nederland, om de prevalentie van deze nieuwe mutatie in *A. fumigatus* in de omgeving te onderzoeken. Resistente TR$_{46}$/Y121F/T289A *A. fumigatus* werd ontdekt bij 6 van de 10 bemonsterde regio’s en bleek genetisch gerelateerd aan resistente isolaten gekweekt uit patiënten, wat de hypothese van de ontwikkeling van resistentie in de omgeving verder onderstrept.

Met de opkomst van azool-resistentie onder *A. fumigatus* in Nederland was er noodzaak om het vóórkomen van dit probleem op wereldwijde schaal te gaan onderzoeken. Om deze reden hebben wij een internationaal screenings programma opgezet om de prevalentie van azool-resistentie wereldwijd te kunnen beschrijven. In *Hoofdstuk 5* worden de resultaten van een prospectieve, multicenter, internationale surveillance gepresenteerd. In totaal werden 3.788 *Aspergillus* isolaten uit 22 centra uit 19 landen gescreend met behulp van de Nederlandse 4-wells azoolplaten. Azool-resistente *A. fumigatus* bleek vaker en meer wijdverspreid voor te komen dan eerder bevestigd, en werd gedetecteerd bij 11 van de 17 deelnemende Europese centra uit 9 verschillende landen. Het prevalentiecijfer van azool-resistentie wereldwijd werd berekend op 3,2%. Het merendeel van de isolaten met de TR$_{34}$/L98H mutatie werd gedetecteerd bij patiënten met acute invasieve aspergillose, in vergelijking met de patiënten met aspergillomen en chronische pulmonale aspergilloseziekte, waar dat niet werd gezien. Azool-resistentie kwam voor bij 5,1% van de patiënten met invasieve aspergillose en werd geassocieerd met een verslechterde outcome. Concluderend is het vóórkomen van azool-resistentie bij *A. fumigatus* dus niet beperkt gebleven tot Nederland en komt op wereldwijde schaal voor. Het handelen rondom ziekte veroorzaakt door azool-resistente *A. fumigatus* moet derhalve worden heroverwogen met bovengenoemde resultaten in het achterhoofd.
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Jan van der Linden
Curriculum Vitae

List of publications


Oral presentations at (inter)national conferences and symposia


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UITNODIGING
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Azole resistance in
Aspergillus: epidemiology
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