Molecular mechanisms of azole resistance in *Aspergillus fumigatus*

Simone Camps
Cover illustrations
from left to right:
- light microscopy of *A. fumigatus*
- DNA strand and the nucleotide sequence of part of the *A. fumigatus cyp51A* gene
- *A. fumigatus* colonies cultured on Sabourad agar plates
- chemical structures of azole compounds

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Molecular mechanisms of azole resistance in *Aspergillus fumigatus*

*Proefschrift*

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door

Simone Maria Theodora Camps
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te Venray
Promotor:
prof. dr. P.E. Verweij

Copromotor:
dr. W.J.G. Melchers

Manuscriptcommissie:
prof. dr. M.G. Netea
prof. dr. N.M.A. Blijlevens
prof. dr. B.J. Zwaan (Wageningen Universiteit)
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Microscopic view of a lung biopsy from a patient with invasive aspergillosis. Blancophor staining (picture: Hein van der Lee).
Chapter 1

General introduction and outline of the thesis
Chapter 1

General introduction

The diverse fungal kingdom contains a collection of spectacularly successful decomposers, symbionts, pathogens, and parasites. Fungi play an important role as degraders of organic matter, as industrial manufacturers of food, antibiotics and enzymes and as the best-characterized eukaryotic model systems for scientific and biomedical research [1]. Of the estimated 1.5 million species of fungi [2], only 100 are known to be regularly involved in human and animal mycoses [3] with *Aspergillus fumigatus*, the focus of this thesis, being one of the most predominant pathogenic fungal species of humans [1].

*Aspergillus fumigatus*

*A. fumigatus* is a saprophytic mold abundantly present in soil and decaying organic matter. It produces large numbers of small (2 to 3 μm) airborne asexual spores (conidia), that can survive a wide range of environmental conditions. Due to the 10,000 to 15,000 liter of air we inhale each day, humans are continuously in contact with spores [4] and it is estimated that we inhale at least several hundred of conidia per day [5]. Despite this constant exposure, most humans do not develop fungal diseases, due to elimination of the fungal spores by the innate immune system [5].

On the other hand, *A. fumigatus* is certainly able to cause disease, often depending on the immune status of the patient [5]. Many infection sites have been described, e.g. the skin, soft tissues, sinuses, central nervous system, peritoneum, kidneys, bones, cardiovascular system, eyes, and gastrointestinal tract [5-6]. For most patients, the main portal of entry and site of infection of *A. fumigatus* is the respiratory tract [5]. In individuals with altered lung function such as asthma and cystic fibrosis patients, it can cause allergic bronchopulmonary aspergillosis, a hypersensitive response to fungal components. Noninvasive aspergilloma occurs in preexisting pulmonary cavities which can be caused by diseases such as tuberculosis. Invasive aspergillosis is the most devastating form of aspergillus disease, and usually targets severely immunocompromised patients [5,7-8]. Those most at risk for this life-threatening disease are individuals with hematological malignancies such as leukemia, solid organ and hematopoietic stem cell transplant patients, patients on prolonged corticosteroid therapy, individuals with genetic immunodeficiencies such as chronic granulomatous disease (CGD), and patients infected with human immunodeficiency virus (HIV) [8-9].
Antifungal therapy

Treatment of aspergillosis may include recovery of the immune status (by reducing immunosuppressive therapy), surgery, and antifungal treatment [5,10]. The availability of new antifungals have revolutionized the field of medical mycology in the past few decades [11], and treatment options for *Aspergillus* include three classes of agents: azoles, polyenes and echinocandins (Figure 1) [1].

Azoles can be divided into two groups: imidazoles and triazoles. Most of the antifungal imidazoles are formulated only for topical use, usually because of toxicity or bioavailability problems that limit their potential as systemic agents [11]. The triazoles (fluconazole, itraconazole, posaconazole, and voriconazole) are the largest class of antifungal agents in clinical use [12]. They function by targeting the ergosterol biosynthetic enzyme lanosterol 14α-demethylase (cytochrome P450), encoded by *cyp51* genes in *A. fumigatus* [1]. Azoles act through a nitrogen atom in the azole ring, which binds to an iron atom in the heme group located in the active site of the enzyme. This inhibits the demethylation of lanosterol, causing a block in the production of ergosterol and the accumulation of 14-α-methyl-3,6-diol, a toxic sterol produced by the Δ-5,6-desaturase encoded by *erg3* (Figure 1A) [1]. With ergosterol depleted and replaced with unusual sterols, the normal permeability and fluidity of the fungal membrane is altered, with secondary consequences for membrane-bound enzymes, such as those involved in cell wall synthesis [12].

Polyenes (amphotericin B) are amphipathic drugs, having both hydrophobic and hydrophilic sides. Polyenes bind to ergosterol to create drug-lipid complexes, which intercalate into the fungal cell membrane to form a membrane-spanning channel (Figure 1B). This causes leakage of cellular contents [1,12]. Although the polyenes have been used in the clinic for over 50 years, the major limitation of their use is host toxicity, such as renal dysfunction, which is likely due to the structural similarities between ergosterol and cholesterol in the mammalian cell membrane. To overcome amphotericin B toxicity, various reformulated versions of the agent have been introduced, which are significantly less toxic than the conventional amphotericin B formulation [1,12].

The echinocandins (anidulafungin, caspofungin, and micafungin) are large lipopeptide molecules that act as noncompetitive inhibitors of (1,3)-β-D-glucan synthase (encoded by *fks1*), an enzyme involved in fungal cell wall synthesis. Echinocandins interfere with this enzyme, resulting in the loss of cell wall integrity and severe cell wall stress on the fungal cell [1] (Figure 1C).
Previous page, Figure 1. Antifungal drugs and their targets [1].

(A) Azoles act by targeting lanosterol 14α-demethylase, causing a block in the production of ergosterol. A toxic sterol produced by Erg3 subsequently accumulates resulting in a severe membrane stress on the cell.

(B) Polyenes function by binding to ergosterol. Drug-lipid complexes are created, which intercalate into the fungal cell membrane to form a membrane-spanning channel. This causes cellular ions to leak out of the cell, destroying the proton gradient and resulting in osmotic cellular lysis.

(C) Echinocandins act as noncompetitive inhibitors of (1,3)-β-D-glucan synthase, and thereby cause a loss of cell wall integrity and severe cell wall stress.

Mechanisms of azole resistance

Azoles are the largest and most widely used class of antifungal drugs. Although *A. fumigatus* is intrinsically resistant to the commonly used azole fluconazole [1], it is generally susceptible to the other azole antifungals (itraconazole, posaconazole, and voriconazole). These antifungals play a very important role in the treatment of disease caused by *A. fumigatus*: voriconazole is the first choice therapy for invasive aspergillosis [6,13], itraconazole is commonly used for the treatment of chronic and allergic conditions [14] and posaconazole has been shown to be effective in preventing invasive aspergillosis in patients with certain hematologic malignancies [15-16]. Furthermore, azoles are the only class of agents available in oral formulation which makes it also suitable for long-term or ambulant therapy for chronic and allergic infections.

The susceptibility of an *A. fumigatus* isolate to azoles and other antifungal agents can be determined using *in vitro* susceptibility testing. Various formats have been developed to test the susceptibility of aspergilli, including the European Committee for Antibiotic Susceptibility Testing (EUCAST) E.DEF 9.1 method [17]. For this susceptibility testing methodology, proposed breakpoints have been published to determine the minimum inhibitory concentration (MIC) boundaries between susceptible and resistant (Table 1) [18].

Since the first documented case of *A. fumigatus* resistance in 1997 [19], many azole-resistant strains of *A. fumigatus* have been isolated and characterized. In addition, mutant isolates in which specific genes were altered, were analyzed. Here, various resistance mechanisms are described.
Alteration of the drug target

Alteration of the drug target (Cyp51) is the principal mechanism of resistance among A. fumigatus isolates [20]. There are two Cyp51 enzymes in A. fumigatus, encoded by cyp51A and cyp51B genes [21]. Although mutations in cyp51B have been described [22-23], none of them has been proven to be related to anazole-resistant phenotype. In contrast, various resistance mutations have been described in cyp51A of clinical isolates; the most frequently characterized (hotspot) mutations are at codons G54 [22,24-27], L98 [28-31], and M220 [23,25-26,32-34]. Also mutations at other codons are described including G138, P216, Y431, G432, G434, and G448 [35-40], although they have not been confirmed by transformation of the altered gene into a wildtype A. fumigatus isolate.

Mutations at codon L98 are always accompanied by a tandem repeat of 34 basepairs in the 5´ upstream region of cyp51A (TR34/L98H), which increases cyp51A expression [28,41]. Notably, it was found that the transformation of the azole-resistant cyp51A open reading frame (L98H) or promoter (TR34) into a susceptible isolate could confer only moderate increases in resistance, but recombinants with both the tandem repeat and the L98H substitution exhibited full resistance [28,41], establishing the importance of the combination of both alterations in azole resistance. Recently also a repeat of 53 bp (TR53) was described in the promoter region of cyp51A. However, transformation into a wildtype isolate did not result in the full azole resistant phenotype as observed in the original TR53 isolate so a yet unknown (additional) mutation was suspected [42]. Furthermore, the presence of a 46 bp tandem repeat was found together with two Cyp51A substitutions (TR46/Y121F/T289A), in a renal transplant patient with invasive aspergillosis [43] also conferring resistance to triazoles.

The three tandem repeats of 34, 46 and 53 basepairs are all located in the same region (Figure 2), indicating the importance of this region in playing a role in azole resistance. Furthermore, a recent study identified a transposon-insertion of 1,822 bp into the promoter region of an azole-resistant clinical isolate, located at 370 bp upstream of the startcodon [36]. The isolate had higher expression levels of cyp51A as well, but the direct association with the insertion is currently unknown and awaits further investigation.

The phenotype of a resistant isolate depends on the particular (cyp51A) resistance mechanism as well as its genetic background. Frequently the activity of more than one azole is affected, often referred to as cross-resistance. In Table 2, the current data linking the phenotype to the genotype is shown for various cyp51A mutations.
Table 1. Proposed interpretative breakpoints [18].

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>MIC (mg/l)</th>
<th></th>
<th></th>
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<tr>
<td></td>
<td>Susceptible</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>&lt;2</td>
<td>2</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>&lt;0.5</td>
<td>0.5</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>&lt;2</td>
<td>2</td>
<td>&gt;2</td>
</tr>
</tbody>
</table>

Table 2. The minimum inhibitory concentrations (MICs) of clinical isolates with various Cyp51A substitutions.

<table>
<thead>
<tr>
<th>Cyp51A substitution</th>
<th>MIC (mg/l)</th>
<th></th>
<th></th>
<th>References</th>
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<tbody>
<tr>
<td></td>
<td>ITZ</td>
<td>POS</td>
<td>VOR</td>
<td></td>
</tr>
<tr>
<td>G54E</td>
<td>&gt;8</td>
<td>0.25-1</td>
<td>0.25-0.5</td>
<td>[22,44]*</td>
</tr>
<tr>
<td>G54R</td>
<td>&gt;8</td>
<td>1</td>
<td>0.12-0.5</td>
<td>[22,24-26,44]</td>
</tr>
<tr>
<td>G54V</td>
<td>&gt;8</td>
<td>ND</td>
<td>0.25-1</td>
<td>[22,44]</td>
</tr>
<tr>
<td>G54W</td>
<td>&gt;8</td>
<td>&gt;8</td>
<td>0.12-0.5</td>
<td>[24,44]</td>
</tr>
<tr>
<td>G138C</td>
<td>&gt;8</td>
<td>1-&gt;8</td>
<td>4-&gt;8</td>
<td>[38-39]</td>
</tr>
<tr>
<td>P216L</td>
<td>&gt;8</td>
<td>1</td>
<td>1</td>
<td>[39]</td>
</tr>
<tr>
<td>M220I</td>
<td>&gt;8</td>
<td>0.5</td>
<td>1</td>
<td>[25-26]*</td>
</tr>
<tr>
<td>M220K</td>
<td>&gt;8</td>
<td>1-&gt;8</td>
<td>1-2</td>
<td>[22-23,32-34,44]*</td>
</tr>
<tr>
<td>M220R</td>
<td>&gt;8</td>
<td>2</td>
<td>2</td>
<td>[45]*</td>
</tr>
<tr>
<td>M220T</td>
<td>&gt;8</td>
<td>0.25-0.5</td>
<td>0.5-1</td>
<td>[22-23,44]</td>
</tr>
<tr>
<td>M220V</td>
<td>&gt;8</td>
<td>0.5-1</td>
<td>1-2</td>
<td>[21-22,44]</td>
</tr>
<tr>
<td>M220W</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>[45]</td>
</tr>
<tr>
<td>Y431C</td>
<td>&gt;8</td>
<td>1</td>
<td>4</td>
<td>[39]</td>
</tr>
<tr>
<td>G432S</td>
<td>&gt;8</td>
<td>0.25</td>
<td>0.38</td>
<td>[35]</td>
</tr>
<tr>
<td>G434C</td>
<td>&gt;8</td>
<td>1</td>
<td>4</td>
<td>[39]</td>
</tr>
<tr>
<td>G448S</td>
<td>&gt;8</td>
<td>0.5-1</td>
<td>&gt;8</td>
<td>[37,39]</td>
</tr>
<tr>
<td>TR_{34}/L98H</td>
<td>&gt;8</td>
<td>0.5-8</td>
<td>2-&gt;8</td>
<td>[28,39,46-47]</td>
</tr>
<tr>
<td>TR_{34}/Y121F/T289A</td>
<td>0.5-&gt;8</td>
<td>0.25-4</td>
<td>&gt;8</td>
<td>[43]*</td>
</tr>
<tr>
<td>TR_{53}</td>
<td>&gt;8</td>
<td>0.25</td>
<td>&gt;8</td>
<td>[42]</td>
</tr>
</tbody>
</table>

ITZ, itraconazole; POS, posaconazole; VOR, voriconazole; ND, not described.

*Also from unpublished data.
Figure 2. Position of the 34, 46 and 53 basepair tandem repeats in the *A. fumigatus* 5' upstream region of the *cyp51A* gene.

Repeated regions are underlined and shaded. For TR\textsubscript{46}, two possibilities exist to obtain a perfect repeat of 46 bp. The 34 bp sequence is located at -322 to -289 bp from the *cyp51A* start codon [28,42-43, unpublished results].
Drug efflux mechanisms

The major mechanism responsible for high-level azole resistance in clinical Candida isolates is overexpression of efflux pumps of the ATP-binding cassette (ABC) family and the Major Facilitator Superfamily (MFS) [48]. Although A. fumigatus contains a large number of these efflux pumps [28,49], isolates overexpressing these pumps as anazole resistance mechanism have rarely been described.

In itraconazole-resistant clinical isolate AF72, the finding of a reduced intracellular accumulation of itraconazole was suggestive for drug efflux pumps in mediating resistance [19]. This isolate has been found to overexpress the atrF gene (an ABC transporter efflux pump) when it was grown in the presence of itraconazole [50]. However, in later reports this isolate turned out to contain the G54E substitution in Cyp51A [22,36,51], which itself is already sufficient for itraconazole resistance to develop [22].

There are a two other reports of efflux pump overexpression, although these have been mutant isolates generated in the laboratory and not clinical isolates [52-53]. In the first study, itraconazole-resistant A. fumigatus mutants obtained by UV mutagenesis were analyzed. Some of them had point mutations at Cyp51A codon G54. A part of the mutants, however, appeared to be resistant possibly due to the overexpression of the efflux pumps mdr3 and mdr4 [52]. The second study included isolates obtained after ten in vitro passages in itraconazole-supplemented medium. As was the case in the UV mutagenesis experiments, several isolates had point mutations in cyp51A related to resistance. Furthermore, most of the mutants showed either constitutive high-level expression or induction upon itraconazole-exposure of mdr3, mdr4, and atrF. Some mutants also showed increased expression levels of mdr1 or mdr2 transporters [53]. Importantly, in both studies there was no evidence linking the elevated expression directly with the itraconazole-resistant phenotype of any of the mutants analyzed [52-53]. Moreover, the increased expression might also represent a stress response and does not necessarily have to be related to resistance [36].

Deletion of the transporter-encoding gene abcA did not show increased susceptibility to azoles and other antifungals [54]. Furthermore, of all the above described genes (atrF, mdr1, mdr2, mdr3, mdr4 and abcA), only mdr1 was found as one of the most expressed genes in microarray experiments upon voriconazole exposure [55]. Taken together, although some transporters have been suggested to reduce azole susceptibility, none of them has been proven to play a direct role in resistance. The question thus remains whether efflux transporters play an active role in clinical resistance in A. fumigatus.
Transcription factor modification

Very little is known regarding transcription factors playing a role in *A. fumigatus* antifungal resistance. In *C. albicans* however, gain-of-function mutations in four transcription factors (Tac1, Mrr1, Cap1, and Upc2), have been linked to increased antifungal resistance [56-61]. Tac1, Mrr1, and Cap1 are associated with increased drug efflux. In contrast, Upc2 is thought to cause resistance through an increase in the *erg11* transcript level (*erg11* is the *cyp51* homologue in Candida) [58-59], demonstrating another mechanism whereby drug target overexpression can occur.

It is unknown whether such mutations occur in transcription factors or their binding sites in *A. fumigatus*. Recent results showed that deletion mutants of SrbA, a transcriptional regulator of the sterol regulatory binding protein (SREBP) family, have an increased susceptibility to fluconazole and voriconazole [62]. A subsequent study showed that SrbA is a direct transcriptional regulator of *cyp51A* and, to a lesser extent, *cyp51B*, being the likely underlying mechanism of the increased susceptibility of the *srbA* deletion mutant [63].

Furthermore, studies of Yap1, the *A. fumigatus* homologue of Cap1 in *C. albicans* (see above) show that a hyperactive, truncated form of this transcription factor attenuates susceptibility of *A. fumigatus* to voriconazole [64]. As is the case in the yeast homologue, truncated Yap1 resistance might be mediated by activating expression of transporter genes [60,65].

Emergence of azole resistance

The first documented case of *A. fumigatus* resistance to an azole was itraconazole resistance, described in 1997 [19]. Although the frequency of azole resistance has remained low for many years [66], in the year 2007, a case series of patients with azole-resistant invasive aspergillosis was reported for the first time [31]. Thirteen isolates were obtained from nine patients with primary invasive aspergillosis or with breakthrough infection while receiving itraconazole or voriconazole. The patients were from six hospitals in The Netherlands and the isolates were obtained between 2002 and 2006. Although there was no apparent epidemiological link between the cases, twelve out of the thirteen isolates (92%) carried the TR\(^{34}/L98H\) resistance mechanism in *cyp51A* [31].

Subsequent studies showed that the prospectively collected fungus culture collection of the Radboud University Nijmegen Medical Centre (The Netherlands) contained an increasing number of azole resistant *A. fumigatus* isolates over time. From the year 2000 onwards, patients were observed annually with an itraconazole-resistant isolate, with a maximum prevalence of 10.1% in 2010 (Figure 3) [29,66-67]. A recent prospective nationwide multicentre surveillance study additionally showed that the emergence of resistance was not limited to a single Medical Centre in Nijmegen, but azole resistance was observed in 6 other University Medical Centres in The Netherlands. Again, most of the itraconazole resistant isolates (90%) were found to possess the TR\(^{34}/L98H\) mutation in the *cyp51A* gene [68].
Emergence of resistance is not only observed in The Netherlands, but also in the United Kingdom. Here, a sudden rise in frequency was observed since 2004 [39,45]. In 2009, the frequency of resistance was as high as 20% of patient cases [45]. Remarkably, the distribution of resistance mechanisms differs between the Dutch and British azole-resistant isolates, with the TR_{34}/L98H substitution being highly dominant in the Dutch isolates and several other \textit{cyp51A} mutations being present in the British isolates [18,29,39,68]. Furthermore, in the United Kingdom, an increase was observed in azole-resistant isolates without \textit{cyp51A} mutations; in 2008/2009, 54% of patients did not have a \textit{cyp51A} mutation known to confer resistance [45]. The reasons for these differences are not clear but might be related to differences in the patient populations from which such isolates were cultured.

\textbf{Figure 3.} Itraconazole resistance among \textit{A. fumigatus} isolates cultured from 1994 to 2011 in Nijmegen (The Netherlands). Prevalence of resistance ranges from 2% of patients in 2000 to as high as 10.1% in 2010 (red line) of the total number of patients with a cultured \textit{A. fumigatus} isolate.
Clinical and environmental route of resistance

Resistance may develop in patients treated with azole antifungal agents. Acquired resistance has especially been described in *A. fumigatus* isolates cultured from patients with aspergilloma and cavitary lung disease [25,39]. This involves infection of the patient with an azole-susceptible isolate that becomes resistant due to the azole pressure during treatment. Until recently, this mode of resistance development, ‘the clinical route of resistance’, was the presumed way of acquired resistance.

Although still controversial [69], a second route of resistance development is believed to occur through environmental azole exposure. The environmental route of resistance is especially taken into account for the TR<sub>34</sub>/L98H resistance mechanism, as a single mechanism of azole resistance was rapidly emerging and found in >90% of resistant clinical isolates from different hospitals in The Netherlands [29,31,68]. The dominance of this single resistance mechanism is difficult to explain by resistance development in individual azole-treated patients, as it might be expected that multiple resistance mechanisms would develop [70]. Indeed, the development of a diversity of cyp51A mutations as well as non-cyp51A mediated resistance was shown in a study by Howard et al, where resistance most likely evolved in infecting isolates within the lungs of the individual patients rather than by resistant infection through the environmental route [39]. Furthermore, spread of resistance by person-to-person transmission is unlikely. The patient will either respond to treatment preventing spread of the isolate, or treatment will fail, the patient dies and spread to other patients will be highly unlikely [70]. Therefore, the dominance of this single resistance mechanism would be more consistent with the acquisition from a common environmental source. This is also in line with the finding of azole-resistant aspergillosis in azole-naïve patients, strongly indicating that resistance does not exclusively develop during azole therapy in the patient. On the other hand, also azole-resistant aspergillosis in patients already receiving azole antifungal agents (as prophylaxis or as treatment for other fungal infections) might be possible, as the patients will be exposed to susceptible and resistant conidia through the environmental route. The resistant conidia will subsequently have a selective advantage, allow germination and cause disease [70].

Indeed, azole-resistant *A. fumigatus* isolates of the TR<sub>34</sub>/L98H genotype were present in the (indoor and outdoor) environment in The Netherlands. They were isolated from air samples from patient rooms, hospital water (filter) samples, soil, seeds and compost. Corresponding to the finding that the majority of clinical resistant isolates was of the TR<sub>34</sub>/L98H genotype, also 86% of the environmental resistant isolates harbored this mechanism of resistance [71]. Subsequent microsatellite typing showed that the TR<sub>34</sub>/L98H environmental isolates generally clustered together with TR<sub>34</sub>/L98H clinical isolates, but apart from the azole-susceptible environmental and clinical isolates. This indicated genetic relatedness and possibly a common ancestor [71]. It is unclear if spread can be prevented, but isolates of the TR<sub>34</sub>/L98H genotype
were also recovered from the environment in Denmark [72] and Norway [29,73], and from patients in various European countries such as Austria, Belgium, Denmark, France, Germany, Italy, Spain and the United Kingdom [28,39,74-75]. Additionally, they were recently also isolated in Asia [76-77].

But why are these isolates already resistant in the environment? It has been hypothesized that environmental resistance could be caused by the use of azole fungicides, also referred to as demethylation inhibitors (DMIs). They are commonly used in the environment, for crop protection and material preservation. Azoles are used to preserve materials such as mattresses, and are also used in paints and coatings. In agriculture, DMIs are used pre-harvest as well as post-harvest to protect the crop from fungal disease and spoilage. In the European Union, the use of azoles in agriculture has gradually increased from the mid-seventies, and up to 49% of the agricultural area is treated annually with azole fungicides [78]. Compared to the amount of azoles used in clinical medicine, the volume of DMIs used in agriculture is approximately 350-fold higher in The Netherlands [66], further underlining the comprehensive use in the environment.

Azoles may persist in soil and water for a long time (the half-life of certain azoles is over one year), thereby also constituting a risk of accumulation [78-82], and possibly giving the naturally occurring A. fumigatus a chance to develop resistance. Plant pathogenic fungi resistant to azoles have been reported frequently [83-88]. Remarkably, repeats and insertions in the 5’ upstream region of cyp51 appear to be a common resistance mechanism in azole resistant plant pathogens [89-94], while never described in species of clinical importance, except for A. fumigatus. The development of resistance in plant pathogens through exposure to azole fungicides as well as the finding of repeats and insertions in cyp51 promoter regions of these pathogens, might indicate that TR34/L98H-mediated resistance in A. fumigatus has been evolved as an unintended side-effect of the use of azole fungicides. Furthermore, rapid emergence of resistance due to a single resistance mechanism was also shown in Penicillium digitatum, the most destructive postharvest pathogen of citrus fruits worldwide. Imazalil fungicide resistance increased from 2% in the year 2000 to 60-84% during 2005-2010. As is the case with A. fumigatus, also a predominant resistance mechanism was found, present in almost 90% of the resistant population [93].

Although several reports are rather optimistic and do not consider agricultural practice as a cause of cross-resistance in clinical medicine [78,95-97], one publication states that extra care should be taken in terms of the application of azoles in agriculture, as long as there is no convincing proof indicating that the massive agricultural use of azoles is absolutely independent from the increasing incidences of resistant human pathogenic fungi [82]. There are few reports showing actual fungicide cross-resistance to medical azoles. The maize pathogen Colletotrichum graminicola, resistant to tebuconazole was also resistant to itraconazole and voriconazole, indicating that azole fungicides are indeed able to induce cross-resistance to
medical azoles [98]. In a study in 2005, 36 out of 150 environmental *A. fumigatus* isolates (24%) had a high itraconazole MIC of ≥2 mg/l. At least some of them additionally showed high MICs for agricultural azoles prochloraz (MIC 16 mg/l) or propiconazole (MIC ≥16 mg/l), giving a first indication that cross-resistance might exist in *A. fumigatus* [97]. The finding that metconazole and tebuconazole, two agricultural fungicides, exhibited activity to susceptible *A. fumigatus* isolates but were less active against the TR$_{34}$/L98H resistant isolates [71], further supports a role for fungicides in resistance development in *A. fumigatus*. 
Outline of the thesis

The thesis focuses on azole resistance in *A. fumigatus*, especially with respect to the molecular basis of resistance. The objectives of this thesis were to discover and investigate new mechanisms of azole resistance in *A. fumigatus*. This was done with the purpose to gain a more complete understanding of the mechanisms that can lead to azole resistance in *A. fumigatus* and to gain more insight into the molecular epidemiology of resistance development, so that our strategies for preventing, diagnosing and treating azole-resistant *A. fumigatus* infections can be improved.

This thesis explores the two main routes of azole resistance development/selection: azole resistance development during medical treatment of individual cases and the emergence of resistance through exposure to fungicides in the environment.

**Section I. The patient route of resistance development**
In chapter 2, an aspergillosis case is described in which multiple azole-resistant profiles were found in individual *A. fumigatus* colonies. We identified two new resistance mechanisms and showed that these were indeed associated with the resistant phenotype. In addition, a review of the literature is given in which all cases with therapy induced azole resistance are described. In chapter 3 azole-resistant isolates from another patient were investigated with the aim to determine the resistance mechanism. A new non-cyp51A mechanism of resistance was discovered using whole genome sequencing technology and sexual crossing experiments.

**Section II. Emergence of resistance through environmental azole exposure**
Chapter 4 describes our results supporting an environmental route of resistance development in *A. fumigatus*. In chapter 5 we investigate in detail the molecular epidemiology of TR34/L98H using genotyping techniques. In chapter 6, a new emerging resistance mechanism is described, that is characterized by lack of activity of voriconazole. We show that this new resistance mechanism has spread across the Netherlands, and probably developed through an environmental route of resistance development.

Overall results and conclusions of the studies in this thesis are discussed and summarized in chapters 7 and 8.
Chapter 1

References


General introduction and outline of the thesis


General introduction and outline of the thesis
A. fumigatus colony growing on Sabouraud agar (picture: Ton Rijs).
Section I

The patient route of resistance development
Microscopic image of *A. fumigatus* stained with lactophenol cotton blue (picture: Ton Rijs).
Rapid induction of multiple resistance mechanisms in *Aspergillus fumigatus* during azole therapy: a case study and review of the literature

Camps SMT, van der Linden JWM, Li Y, Kuijper EJ, van Dissel JT, Verweij PE, Melchers WJG

Antimicrobial Agents and Chemotherapy, 2012;56(1):10-16
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 [1-3]. 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 [2,4-5], which is supported by experimental models of aspergillosis [6-11]. Surveillance studies indicate that the prevalence of azole resistance varies widely between countries [12-14], and there is increasing evidence that in addition to patient therapy, environmental exposure to azole compounds may be an important route of resistance development [15-16]. The most common mechanisms of resistance in *A. fumigatus* are modifications in the *cyp51A* gene [17]. *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 [18-19].

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 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 switched 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 1 month of treatment with L-AMB and CAS, the patient had ongoing *Aspergillus* disease and died because of a 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) [20] as part of a national surveillance study [21]. 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 [22]. ITC, POS, VRC, and amphotericin B (AMB) were assayed over a 2-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 [14].

*Cyp51* 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 ~0.1 g glass beads (diameters, 0.4 to 0.6 mm). After shaking by vortexing, conidia were incubated at 70°C for 30 min while shaking.
Then, 200 μl of phenol-chloroform-isooamyl 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 [23]. The promoter region was amplified using previously described primers P-A7 and P-A5 [24] and sequenced using the forward primer (P-A7). The cyp51B gene and promoter region were amplified and sequenced using primer sets 5'-CTTTATTCCTGCAGACA-3'/ 5'-ACGGCAGATACCCAGA-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 (STRAf 3A, 3B, 3C, 4A, 4B, and 4C) were amplified as described before [25]. The sizes of the fragments were determined, and repeat numbers were assigned [15]. Genotypes consisting of the number of repeats for each of the six microsatellites were created for all isolates.

**Cyp51A sequence alignment and homology model**

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 [23].

**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′-CTGGACAAGGCTTTACTCTCATCAATTTATGCTACCG-3′/5′-CGGTAGCATAAAATTGATGAGAGTAAAGCCCTTGTCCAG-3′ (for P216L) and 5′-GCTTTACTCCCATCAATATTATGCTACCGTGGGCC-3′/5′-GGCCCACGGTAGCATAATATTGATGGGAGTAAAGC-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 profiles 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.

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. 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 [27], 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).
Table 1. Isolates obtained from the patient suffering from pulmonary aspergilloma.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Date of isolation</th>
<th>Specimen</th>
<th>Cyp51A substitution</th>
<th>MIC (mg/liter)</th>
<th>Microsatellite no. of repeats</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ITC</td>
<td>VRC</td>
<td>POS</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>
<td>0.063</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>
<td>0.5</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>
<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>
<td>&gt;16</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>
<td>&gt;16</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>
<td>&gt;16</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>
<td>&gt;16</td>
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<tr>
<td>v83-11</td>
<td>5-6-2009</td>
<td>Sputum</td>
<td>A9T, F219I</td>
<td>&gt;16</td>
<td>4</td>
<td>&gt;16</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>
<td>1</td>
</tr>
</tbody>
</table>

Next page, 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 F219 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.
## Induction of resistance during azole therapy

<table>
<thead>
<tr>
<th>Yeast</th>
<th>A9</th>
<th>G54</th>
<th>P216</th>
<th>F219</th>
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<td>C. albicans</td>
<td><em><strong>SLSVTQISIL</strong></em>SAASYQPYETEDKGFTEINVFNL**</td>
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<td>C. tropicalis</td>
<td><em><strong>SLSLQITIL</strong></em>SAIPFMAPYETEDKGFTEINVFNL**</td>
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<td>S. stipitis</td>
<td><em><strong>SLSLTQVSI</strong></em>SAASYQPYETEDKGFTEINVFNL**</td>
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<td>C. lusitaniae</td>
<td><em><strong>SLGLKKIALL</strong></em>SAVVYQPYETEDKGFTEINVFNL**</td>
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<td>C. krusei</td>
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<td>C. glabrata</td>
<td><em><strong>ALPLAQVSI</strong></em>SAIPFTQPYETEDKGFTEINVFNL**</td>
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<tr>
<td>S. cerevisiae</td>
<td><em><strong>ALPLAQVSI</strong></em>SAIPFTQPYETEDKGFTEINVFNL**</td>
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<tr>
<td>Z. rouxii</td>
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<td>E. gossypii</td>
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<td>Y. lipolytica</td>
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<td>A. fumigatus Cyp51A</td>
<td><em><strong>MLWLTAYMAVA</strong></em>STISYGIDPYETEDKGFTEINMLPWA**</td>
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<td><em><strong>MLLTLTAYMAVA</strong></em>STISYGIDPYETEDKGFTEINMLPWA**</td>
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<td><em><strong>LTLFGLVLSI</strong></em>STISYGIDPYETEDKGFTEINMLPWA**</td>
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<td>A. flavus Cyp51A</td>
<td><em><strong>TFLGTTYVSGL</strong></em>STISYGIDPYETEDKGFTEINMLPWA**</td>
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<td>A. nidulans</td>
<td><em><strong>PTLFSYTVGL</strong></em>STISYGIDPYETEDKGFTEINMLPWA**</td>
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<td>P. italicum</td>
<td><em><strong>TGLIAVTTGL</strong></em>STISYGIDPYETEDKGFTEINMLPWA**</td>
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<td>B. graminis</td>
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<td>O. acuformis</td>
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<td>B. jaapii</td>
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<td>M. fructicola</td>
<td><em><strong>GTIAAYGVA</strong></em>STISYGIDPYETEDKGFTEINMLPWA**</td>
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<td>B. fuckeliana</td>
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<td>N. crassa</td>
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<td>L. maculans</td>
<td><em><strong>FVLAILGFAA</strong></em>STISYGIDPYETEDKGFTEINMLPWA**</td>
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<td>N. fischeri Cyp51B</td>
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<td><em><strong>WMLGGGLLSL</strong></em>STISYGIDPYETEDKGFTEINMLPWA**</td>
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<td>A. flavus Cyp51B</td>
<td>-------------------------------STISYGIDPYETEDKGFTEINMLPWA**</td>
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<td>P. digitatum Cyp51B</td>
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<td>P. marneffei Cyp51B</td>
<td><em><strong>YVLAGSFCAVF</strong></em>NTISYGIDPYETEDKGFTEINMLPWA**</td>
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<td>C. posadasii</td>
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<td>A. capsulatus</td>
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<td>V. inaequalis</td>
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<td>V. nashicola</td>
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<td>M. graminicola</td>
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<td>S. pombe</td>
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<td>P. carinii</td>
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<td>C. neoformans</td>
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<td>T. asahii</td>
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<td>P. chrysosporium</td>
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<td>U. maydis</td>
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<td>C. elegans</td>
<td><strong>---TIISSYSVLA</strong><em>NAVQFTMNPSA</em><strong>STISYGIDPYETEDKGFTEINVFNL</strong></td>
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</table>
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.

_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 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.
Table 2. MICs of recombinants with either the P216L or F219I substitution compared to those of the recipient isolate and the transformation control isolate.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Base change in cyp51A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC (µg/ml)</th>
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<tr>
<td></td>
<td>codon 216</td>
<td>codon 219</td>
<td>ITC</td>
</tr>
<tr>
<td>Transformation recipient isolate</td>
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<td>TTT</td>
<td>0.5</td>
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<tr>
<td>Transformation control</td>
<td>CCC</td>
<td>TTT</td>
<td>0.5</td>
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<tr>
<td>Recombinant P216L</td>
<td>CTC</td>
<td>TTT</td>
<td>&gt;16</td>
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<tr>
<td>Recombinant F219I</td>
<td>CCC</td>
<td>ATT</td>
<td>&gt;16</td>
</tr>
</tbody>
</table>

<sup>a</sup> Bold indicates the base change.

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 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).

Next page, Figure 3. Reported cases of acquired azole resistance in *A. fumigatus* [1-2,6,28-29].

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 F219I 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).
this study
Induction of resistance during azole therapy

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* [1,27,30-32] and are even mentioned to be hot-spot mutations [33] 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 [27,30-31]. 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 [2]. 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 [19], 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 [2,28,34].

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 to azole 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 [16].

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) received azole 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.

**Acknowledgements**

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


Paired culture of two *A. fumigatus* isolates of opposite mating type.
Cleistothecia arise along the junctions of intersecting colonies (picture: Ton Rijs and Simone Camps).
Discovery of a *hapE* mutation that causes azole resistance in *Aspergillus fumigatus* through whole genome sequencing and sexual crossing

Camps SMT*, Dutilh BE*, Arendrup MC, Rijs AJMM, Snelders E, Huynen MA, Verweij PE, Melchers WJG


*contributed equally to this publication
Abstract

Azole compounds are the primary therapy for patients with diseases caused by *Aspergillus fumigatus*. However, prolonged treatment may cause resistance to develop, which is associated with treatment failure. The azole target *cyp51A* is a hotspot for mutations that confer phenotypic resistance, but in an increasing number of resistant isolates the underlying mechanism remains unknown. Here, we report the discovery of a novel resistance mechanism, caused by a mutation in the CCAAT-binding transcription factor complex subunit HapE. From one patient, four *A. fumigatus* isolates were serially collected. The last two isolates developed an azole resistant phenotype during prolonged azole therapy. Because the resistant isolates contained a wild type *cyp51A* gene and the isolates were isogenic, the complete genomes of the last susceptible isolate and the first resistant isolate (taken 17 weeks apart) were sequenced using Illumina technology to identify the resistance conferring mutation. By comparing the genome sequences to each other as well as to two *A. fumigatus* reference genomes, several potential non-synonymous mutations in protein-coding regions were identified, six of which could be confirmed by PCR and Sanger sequencing. Subsequent sexual crossing experiments showed that resistant progeny always contained a P88L substitution in HapE, while the presence of the other five mutations did not correlate with resistance in the progeny. Cloning the mutated *hapE* gene into the azole susceptible *akuB*KU80 strain showed that the HapE P88L mutation by itself could confer the resistant phenotype. This is the first time that whole genome sequencing and sexual crossing strategies have been used to find the genetic basis of a trait of interest in *A. fumigatus*. The discovery may help understand alternate pathways for azole resistance in *A. fumigatus* with implications for the molecular diagnosis of resistance and drug discovery.
Introduction

*Aspergillus fumigatus* is a ubiquitous saprophytic mold. Although humans inhale at least several hundred of *A. fumigatus* conidia per day, it rarely causes serious medical conditions in healthy individuals. In contrast, immunocompromised patients such as solid organ and hematopoietic stem cell transplant recipients and patients receiving chemotherapy are at risk of developing invasive aspergillosis, a commonly fatal infection [1]. Additionally, *A. fumigatus* is able to cause a wide range of other non-invasive diseases including allergic syndromes and aspergilloma, many of which require treatment with antifungal agents [1,2].

Current treatment options of *Aspergillus* diseases include three classes of antifungal agents: polyenes (amphotericin B), echinocandins (caspofungin) and azoles, the latter being the largest and most widely used class of antifungal drugs [3,4]. Voriconazole is the recommended first choice therapy for invasive aspergillosis [5,6]. Itraconazole is commonly used for the treatment of chronic and allergic conditions [2] and posaconazole is effective in preventing invasive aspergillosis in patients with certain hematologic malignancies [7,8]. Although *A. fumigatus* is generally susceptible to these azole antifungals, acquired resistance is increasingly being reported over the last few years [9-12]. Evidence is also accumulating that patients suffering from azole-resistant *Aspergillus* disease may fail to respond to therapy [11,13-19]. The most common mechanisms of resistance in *A. fumigatus* are mutations in the *cyp51* gene encoding sterol 14α-demethylase, the target for azoles. Azoles inhibit this enzyme, thereby blocking its function in the ergosterol biosynthesis pathway, resulting in ergosterol depletion and accumulation of toxic sterols [20]. The *A. fumigatus* genome contains two distinct but closely related *cyp51* (*erg11*) genes: *cyp51A* and *cyp51B*, each encoding a different protein [21]. Mutations have rarely been detected in the *cyp51B* gene and have never been shown to be related to azole resistance [4]. In contrast, certain non-synonymous point mutations in the *cyp51A* gene (for example at codons G54, G138 and M220) correspond with azole resistance [22-24]. Other mutations responsible for azole resistance include tandem repeats of 34, 46 and 53 bp in the *cyp51A* promoter region (generally combined with mutations in the gene itself), resulting in an increased expression of the *cyp51A* gene [13,25-27]. In addition, an *Aft1* transposon was found inserted 370 bp upstream of the start codon, possibly modulating *cyp51A* expression as well [28].

As is the case for *A. fumigatus*, azole resistance in other fungi such as *Candida* may be caused by alterations and over-expression of the azole target 14α-demethylase. Alternatively, over-expression of drug efflux transporters that pump the toxic azoles out of the cell have also been related to resistance in *Candida* [29,30]. *A. fumigatus* contains several efflux pumps of the ATB-binding cassette (ABC) family and the Major Facilitator Superfamily (MFS) [31]. Although some of these transporters have been suggested to reduce azole susceptibility
Chapter 3

[31-35], none of them has been proven to play a direct role in resistance yet [28]. The question thus remains whether efflux transporters play an active role in azole resistance in \textit{A. fumigatus} [34,36]. An alternative azole resistance mechanism in \textit{Candida} is a defective sterol Δ5,6-desaturase, encoded by the \textit{erg3} gene. This allows the accumulation of less toxic sterols in the presence of azole antifungals [29,31,37]. However, the \textit{erg3} genes have never been implicated in azole resistance in \textit{A. fumigatus} [37].

Today, an increasing number of azole-resistant isolates are being reported without alterations in the \textit{cyp51A} gene or promoter region [9]. However, no alternate resistance mutations have yet been found in clinical \textit{A. fumigatus} isolates. Attempts to find new genes involved in azole resistance included determining expression levels of specific transporter genes [33-35,38] and disruption, duplication, or truncation of a specific gene of interest followed by analysis of antifungal susceptibility in the recombinant [39-45]. Here, we apply a new strategy to unravel the resistance mechanism using a set of four sequentially isolated isogenic clinical \textit{A. fumigatus} isolates that acquired an azole resistant phenotype during prolonged treatment. The isolates were obtained from a patient with chronic granulomatous disease (CGD) as described in detail in a previous study [46]. The first two isolates were susceptible to azole antifungals, but the last two isolates were azole resistant and the patient failed azole-echinocandin combination therapy. As the \textit{A. fumigatus} isolates did not contain any mutations in the \textit{cyp51A} gene or its promoter region, this set of isolates offered a unique opportunity to study the \textit{de novo} acquisition of non-\textit{cyp51A} azole resistance mutations. Our strategy included whole genome sequencing, sexual crossing experiments and gene replacements to identify the gene responsible for the observed resistance.

Materials and methods

\textbf{Origin and characterization of \textit{A. fumigatus} isolates}

The patient, origin of the isolates, and characterization of the isolates were described in detail in a previous publication [46]. The patient was a 21 year-old male with CGD. Due to a pulmonary infection caused by \textit{A. fumigatus}, he was treated with multiple courses of antifungal therapy including caspofungin, voriconazole and a combination of the two. After relapse of the infection half a year later various treatment regimens were implemented including voriconazole, caspofungin/voriconazole combination therapy and caspofungin/posaconazole combination therapy. During this relapsing infection various respiratory samples were taken under routine care and four \textit{A. fumigatus} isolates were obtained, designated isolates 1 through 4 (Figure 1). Unfortunately, despite antifungal treatment it was not possible to eliminate the fungus and the patient died from his pulmonary infection. \textit{In vitro} susceptibility testing of the patient isolates was performed using the EUCAST broth
A hapE mutation that causes azole resistance

microdilution reference method [47]. Results were interpreted using the previously proposed breakpoints [12]. DNA was isolated and STRAf 3A, 3B, 3C, 4A, 4B, and 4C microsatellite genotypes were determined [48]. The cyp51A and cyp51B coding genes and promoter regions were amplified and sequenced as described before [48]. Sequences of the four patient isolates were aligned to identify any sequence differences in cyp51A and cyp51B.

Complete genome sequencing and assembly
In order to unravel the resistance mechanism, isolates 2 and 3 (isolated 17 weeks apart, directly before and after the onset of azole resistance) were selected for complete genome sequencing (Figure 1). The isolates were cultured in liquid medium and after harvesting and drying mycelial mats, DNA was isolated as described elsewhere [49]. The Illumina Paired-End DNA sequencing Sample Prep Kit (cat #1001809) was used to process the DNA samples. Fragmentation of the DNA by nebulization, ligation of sequencing adapters and PCR amplification of the resulting product was performed according to the Illumina protocol Preparing Samples for Paired-end Sequencing (1005063 Rev. A). The quality and yield after sample preparation was measured with Lab-on-a-Chip analysis. The size of the resulting product was consistent with the expected size of approximately 300 bp after excision from an agarose gel. DNA sequencing using the Genome Analyzer II (Illumina) was performed according manufacturer’s protocols. Two sequencing reads of 36 cycles each using the Read 1 sequencing and Read 2 sequencing primers were performed with the flow cell. Sequencing data are available at the Sequence Read Archive (SRA) at http://www.ebi.ac.uk/ena/data/view/ under accession number ERP001097. The two complete A. fumigatus genomes that are publically available (clinical isolates Af293 and A1163 [50,51]) were used as reference sequences to aid the genome assembly. Assembly was done using Maq [52] with default parameters, taking into account the paired-end nature of the reads.
Figure 1. Relapsing infection with *A. fumigatus* in a CGD patient. During the course of the disease, four *A. fumigatus* isolates were obtained. Sampling dates of isolates 1 through 4 are indicated. S indicates susceptibility to azoles and R indicates resistance. Minimum inhibitory concentrations (MICs) for itraconazole, voriconazole, and posaconazole are also shown. The patient was treated with voriconazole monotherapy (week -67 to +12), caspofungin + voriconazole combination therapy (week +12 to +90) and caspofungin + posaconazole combination therapy (week +90 to +134). Plasma concentrations at week 86 (voriconazole) and weeks 99, 105, and 123 (posaconazole) are also indicated. The patient died from the pulmonary infection at week 134 [46].

<table>
<thead>
<tr>
<th>Isolate:</th>
<th>MIC itraconazole (mg/l):</th>
<th>MIC voriconazole (mg/l):</th>
<th>MIC posaconazole (mg/l):</th>
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<tr>
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<td>1</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>128</td>
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<table>
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<th>Week:</th>
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<td>3</td>
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<td>0.5 0.5</td>
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</table>
Prioritization and validation of potential mutations

From the complete genome sequencing data, non-synonymous mutations were selected in isolate 3 (resistant) relative to isolate 2 (susceptible) and both reference isolates Af293 and A1163 (susceptible). Many of these mutations were expected to be unreliable because of low sequencing depth or low quality of the Maq consensus assembly. Potential mutations were prioritized based on the product of the Maq assembly quality scores in the susceptible and resistant assembly (further referred to as ‘product quality score’). As we did not know *a priori* what cutoffs to use, mutations with the highest product quality scores were selected for confirmation by conventional PCR and Sanger sequencing. Moreover, potential mutations with lower product quality scores were also validated for proteins potentially important in azole resistance (e.g. transporters), or if they occurred in the genomic vicinity of more reliably called mutations and could be validated in one sequencing experiment. For the selected mutations, primers were designed (Table 1) and used to amplify and sequence isolates 2 and 3 first. For confirmed mutations, isolates 1 and 4 were subsequently amplified and sequenced.

Sexual reproduction and analysis of the progeny

The mating type of the isolates was determined as described before [53]. Isolates 1, 2, 3 and 4 were each crossed with isolate 47-169, a highly fertile environmental *A. fumigatus* isolate (kindly provided by C.M. O’Gorman and P.S. Dyer, School of Biology, University of Nottingham, Nottingham, UK). Mating experiments were performed on oatmeal agar plates (Difco Oatmeal agar) and inoculation was performed as described previously [54]. Plates were sealed with Parafilm and incubated at 30°C in the dark. Crosses were examined weekly and when cleistothecia developed, ascospore suspensions were obtained as described before [54]. The suspensions were heated at 70°C for one hour to prevent the germination of asexual spores [54]. Then small aliquots of the suspensions were plated onto Sabouraud agar plates and incubated for two days at 28°C. Germinating ascospores were transferred to Sabouraud agar slants and grown at 28°C for five days. The progeny were tested for their susceptibility to azoles as described above and DNA was isolated as described elsewhere [48]. The DNA was used to check for the presence or absence of the confirmed mutations by PCR and sequencing, using the same primers as used for validation (Table 1). In addition, from a selection of the progeny the microsatellite genotype was determined [48].
<table>
<thead>
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<td>CCTGTCAGGTTAGTGCCTT</td>
<td>incorrect</td>
</tr>
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<td></td>
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<td>411.30</td>
<td>AGGAACGTCTATGGGATAC</td>
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<td>69</td>
<td>AtrD</td>
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<td>411.18</td>
<td>GTCTGCTCTGCTGCTG</td>
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</table>
A *hapE* mutation that causes azole resistance

**Previous page, Table 1.** Validation of potential mutations identified in the complete genome sequence of isolate 3 relative to isolate 2 as well as both reference strains. Potential mutations were prioritized using the product quality score (see Methods). Primers used for amplification and sequencing are indicated. The nine highest quality scores (above ‘…..’) as well as several mutations with lower scores (below ‘…..’) were validated. ‘Correct’ indicates that the mutation found by complete genome sequencing was validated using conventional PCR and sequencing methods; ‘incorrect’ indicates isolate 3 did not differ from isolate 2.

**Growth kinetic assay**

Part of the progeny was selected to evaluate growth over time. For that, conidia were suspended in 0.1% Tween-20. Suspensions were adjusted to 1-4.2*10^6 conidia/ml and diluted 1:10 in water. 100 µl thereof was added to 100 µl of double strength RPMI 2% glucose medium in three individual wells of a microtiter plate. The plates were incubated at 37°C for 12 h inside a plate reader. The optical density (OD) at 450 nm was automatically recorded for each well every 30 minutes without shaking. For each time point the OD of the triplicate was averaged and the changes in OD over time were used to generate growth curves. The maximum growth per hour during the exponential section of the curve was averaged for the susceptible and resistant progeny and to test whether differences were significant, student’s t-test was applied (one-tailed, heteroscedastic).

**RNA isolation and cyp51A expression**

A selection of the progeny and parental isolates were subjected to cyp51A expression analysis. The isolates were cultured twice and used for separate total RNA isolation. cDNA amplification and RT-PCR for cyp51A and actin expression levels were performed as described before [46]. The change in gene expression was determined using the cyp51A/actin ratio.

**Transformations**

Gene replacement experiments were performed as described before [22,26]. Briefly, DNA fragments of the six genes with mutations (Table 2) were obtained by PCR amplification using genomic DNA of the first resistant isolate 3. As a positive control, a fragment with a mutation in cyp51A known to be correlated with azole resistance (the cyp51A G54E substitution in clinical isolate AF-72 [22]), was amplified. The primers used to amplify the six genes as well as the cyp51A gene for the positive control are shown in Table 2. All primers were designed to include the complete gene, except for alpha-aminoadipate reductase large subunit (reference Af293 gene AFUA_4G11240 and A1163 gene AFUB_068270). Because of the large size of this gene, primers were designed approximately 1000bp on both sides of the mutation. The PCR products were used for homologous gene replacement by electroporation into the azole susceptible isolate *akuB* [26,55]. Transformation mixtures were spread-plated on minimal-medium plates containing itraconazole (0.5 to 4 mg/l) to select for resistant recombinants.
After three days of incubation at 37°C colonies were subcultured for further investigation. DNA was isolated and the recombinants were analyzed for the presence of the mutation by using the corresponding primers listed in Table 1. In successful recombinants, the complete mutant gene (primers Table 2) and the cyp51A gene and promoter region (as described above) were sequenced to ensure no other mutations were introduced. The susceptibility profiles of the successful recombinants (two recombinants were selected for each mutation) and the transformation recipient isolate akuB<sup>KU80</sup> were determined as described above.

<table>
<thead>
<tr>
<th>Gene (abbreviated)</th>
<th>Gene ID reference isolates Af293 / A1163</th>
<th>Primer ID</th>
<th>Forward / reverse primer</th>
<th>Primer sequence (5’-3’)</th>
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<tr>
<td>hypothetical</td>
<td>AFUA_2G02180 / AFUB_019270</td>
<td>411.55</td>
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<td></td>
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<td>411.56</td>
<td>reverse</td>
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<td>AFUA_4G03630 / AFUB_099400</td>
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<td>CGTACCTTTACTGACACTTTGAA</td>
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<td>411.54</td>
<td>reverse</td>
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<td>365.1</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>365.2</td>
<td>reverse</td>
<td>CTGTCGACTTTGATGG</td>
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</table>

**HapE sequencing in resistant isolates without cyp51A mutations**

Our fungal culture collection contains *A. fumigatus* clinical isolates from patients admitted to our hospital, as well as environmental isolates and isolates sent to our laboratory for identification, *in vitro* susceptibility testing, or research purposes. Only accredited scientists in our laboratory have access to the fungal collection. The collection was searched for isolates resistant to one or more azoles but without any cyp51A gene mutations known to be involved in resistance. Of these isolates *hapE* was amplified by PCR and subsequently sequenced (primers Table 2). Gene sequences were compared with isolate 2 to detect any mutations.
Results

Characterization of the isolates

The azole minimum inhibitory concentrations (MICs) of the four isolates obtained during the course of the disease in a CGD patient are shown in Figure 1. According to the suggested breakpoints [12], isolates 1 and 2 were azole susceptible, while isolate 3 and 4 were resistant to both itraconazole and voriconazole and had an intermediate susceptibility to posaconazole. The isolates did not have any nucleotide differences in their cyp51A and cyp51B genes and promoter regions (results not shown) and microsatellite genotyping showed that the four isolates had identical genotypes and were thus isogenic (results not shown).

Genome sequencing, assembly and validation of potential mutations

As no cyp51A and cyp51B mutations developed, a new, unknown resistance mutation must have emerged during therapy. Due to the isogenic nature of the isolates, we anticipated that the most straightforward approach to identify this resistance mechanism would be by complete genome sequencing of isolates 2 and 3 (isolated directly before and after the onset of azole resistance). The volume of sequence data obtained using Illumina technology included 8,611,525 36-nt read pairs for isolate 2 and 4,808,633 36-nt read pairs for isolate 3. The total sequence volume was 620,029,800 nt (isolate 2) and 346,221,576 nt (isolate 3). After reference-guided assembly, the sequence coverage of isolate 2 was 93.7% (mapped against Af293) and 94.9% (mapped against Af1163). For isolate 3 this was 93.6% (mapped against Af293) and 94.8% (mapped against Af1163). The average sequencing depth was 20.3 reads for isolate 2 and 11.5 for isolate 3. Sixty-nine potential mutations were identified in resistant isolate 3 that were non-synonymous relative to both the susceptible isolate 2 and both reference isolates Af293/A1163 (Table S1, available at http://dx.plos.org/10.1371/journal.pone.0050034). The potential mutations were then prioritized based on their product quality scores. Mutations with the highest scores as well as a selection of genes with lower scores were validated by PCR and Sanger sequencing. Table 1 shows that only the six mutations with the highest product quality scores could be confirmed, the other mutations were false-positive calls. Subsequent testing of isolate 1 and 4 confirmed the presence of these six non-synonymous mutations in both resistant isolates 3 and 4 relative to the susceptible isolates 1 and 2 (Table 3).

Sexual reproduction and analysis of the progeny

To elucidate which of the six mutations was the cause of resistance, sexual crosses were performed. Note that this approach would also identify potential combinations of mutations if two or more mutations were together responsible for the resistant phenotype. Isolates 1 to 4 were of mating type MAT1-2 and were crossed with 47-169, an isolate of the opposite
mating type MAT1-1. After six weeks of incubation, cleistothecia appeared on the agar plate. Ascospore progeny were isolated and subcultured. Ten (for isolates 1 and 2), forty (for isolate 3) and twenty (for isolate 4) progeny isolates were randomly selected for susceptibility testing and azole resistant isolates were present in the progeny of isolates 3 and 4, but not in the progeny of isolates 1 and 2 (Table 4). The resistant progeny all exhibited a MIC of >16 mg/l for itraconazole. In addition, these isolates showed elevated MICs for voriconazole and posaconazole, similar to those observed in patient isolates 3 and 4 (results not shown). In addition, a minority of the progeny of the resistant isolates 3 and 4 exhibited an altered susceptibility phenotype for itraconazole, referred to as trailing effect [56].

The forty progeny of isolate 3 were screened for the presence of the six mutations validated in the whole genome sequencing analysis. All mutations were present in the susceptible as well as in the resistant progeny, except for the mutation in the hapE gene which was only present in resistant isolates. Furthermore, Table 5 shows that all resistant progeny of isolate 3 and 4 contained the hapE mutation while the other mutations were only present in a part of the resistant progeny. Microsatellite typing (Table 6) showed that twelve out of the thirteen resistant progeny (92%) had a unique microsatellite genotype, indicating that unique germinating ascospores harboring the resistance mutation were isolated and not a single clone of resistant progeny. Taken together, these results strongly suggest that the mutation in hapE caused the observed resistant phenotype.

Sixteen of the progeny as well as both parental isolates (isolate 3 and 47-169) were selected to determine in vitro growth curves (Figure S1, available at http://dx.plos.org/10.1371/journal.pone.0050034). As can be observed in Figure 2, growth rate for susceptible progeny was significantly higher compared to the resistant progeny (p=0.015). In order to determine if the cyp51A expression level of progeny containing the HapE substitution was altered, RT-PCR experiments were performed. A ~1.5-fold induction of the transcriptional profile of the hapE-containing progeny isolate compared to two susceptible progeny isolates was found (Figure 3).
A *hapE* mutation that causes azole resistance

**Table 3.** The six confirmed non-synonymous mutations, together with results of complete genome sequencing and validation.

<table>
<thead>
<tr>
<th>Gene function</th>
<th>Protein ID AF293 / A1163</th>
<th>Nucleotide in complete genome sequence isolate 2 / 3</th>
<th>Validated nucleotide in isolate 1 / 2 / 3 / 4</th>
<th>Amino acid substitution</th>
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<tr>
<td>Conserved hypothetical protein</td>
<td>AFUA_2G02180 / AFUB_019270</td>
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<td>C / C / T / T</td>
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<td>Sterol 24-c-methyltransferase, putative (Erg6)</td>
<td>AFUA_4G03630 / AFUB_099400</td>
<td>T / G</td>
<td>T / T / G / G</td>
<td>W320G</td>
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<tr>
<td>CCAAT-binding factor complex subunit HapE</td>
<td>AFUA_6G05300 / AFUB_092980</td>
<td>C / T</td>
<td>C / C / T / T</td>
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<td>G / G / A / A</td>
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<td>Alpha-aminoadipate reductase large subunit, putative</td>
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<td>T / T / C / C</td>
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<td>C2H2 finger and ankyrin domain protein, putative</td>
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<td>T / T / A / A</td>
<td>Y347*</td>
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* refers to a premature stop codon.

**Table 4.** Distribution of susceptible and resistant isolates in the sexual crossing progeny of isolate 47-169 and each of the four isolates in this study.

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<th>Phenotype</th>
<th>Progeny isolate 1</th>
<th>Progeny isolate 2</th>
<th>Progeny isolate 3</th>
<th>Progeny isolate 4</th>
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<td>10 (100%)</td>
<td>10 (100%)</td>
<td>25 (63%)</td>
<td>11 (55%)</td>
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<td>Trailing-endpoint itraconazole</td>
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<td>-</td>
<td>8 (20%)</td>
<td>3 (15%)</td>
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<tr>
<td>Resistant</td>
<td>-</td>
<td>-</td>
<td>7 (18%)</td>
<td>6 (30%)</td>
</tr>
<tr>
<td>Total</td>
<td>10 (100%)</td>
<td>10 (100%)</td>
<td>40 (100%)</td>
<td>20 (100%)</td>
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</table>
Table 5. Resistant progeny of crosses 47-169 x isolate 3 and 47-169 x isolate 4: results of susceptibility testing and mutation screening.

<table>
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<th>Isolate</th>
<th>MIC (mg/l)</th>
<th>Gene&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>ITZ</td>
<td>VOR</td>
<td>POS</td>
<td>hypoth.</td>
<td>erg6</td>
<td>hapE</td>
<td>erg25</td>
<td>alpha</td>
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<td>0.5</td>
<td>0.063</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>isolate 3 parent</td>
<td>&gt;16</td>
<td>4</td>
<td>0.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>isolate 4 parent</td>
<td>&gt;16</td>
<td>4</td>
<td>0.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>0.5</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>v121-54 progeny</td>
<td>&gt;16</td>
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<td>0.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>v121-55 progeny</td>
<td>&gt;16</td>
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<td>0.5</td>
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<td>+</td>
<td>+</td>
<td>-</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>v121-74 progeny</td>
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<td>v121-78 progeny</td>
<td>&gt;16</td>
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<td>0.5</td>
<td>-</td>
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<td>+</td>
<td>-</td>
<td>+</td>
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<td>v122-02 progeny</td>
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<tr>
<td>v122-08 progeny</td>
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<tr>
<td>v122-12 progeny</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>v122-13 progeny</td>
<td>&gt;16</td>
<td>4</td>
<td>0.5</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>v122-17 progeny</td>
<td>&gt;16</td>
<td>2</td>
<td>0.25</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>v122-18 progeny</td>
<td>&gt;16</td>
<td>2</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> ‘−’ indicates absence of the mutation, ‘+’ indicates presence of the mutation.
### Table 6. Microsatellite typing results of the resistant progeny.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Microsatellite genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3A</td>
</tr>
<tr>
<td>47-169 parent</td>
<td>16</td>
</tr>
<tr>
<td>isolate 3 parent</td>
<td>47</td>
</tr>
<tr>
<td>isolate 4 parent</td>
<td>47</td>
</tr>
<tr>
<td>v121-42 progeny (47-169 x isolate 3)</td>
<td>16</td>
</tr>
<tr>
<td>v121-43 progeny (47-169 x isolate 3)</td>
<td>16</td>
</tr>
<tr>
<td>v121-54 progeny (47-169 x isolate 3)</td>
<td>16</td>
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<tr>
<td>v121-55 progeny (47-169 x isolate 3)</td>
<td>16</td>
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<tr>
<td>v121-59 progeny (47-169 x isolate 3)</td>
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</tr>
<tr>
<td>v121-74 progeny (47-169 x isolate 3)</td>
<td>16</td>
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<tr>
<td>v121-78 progeny (47-169 x isolate 3)</td>
<td>16</td>
</tr>
<tr>
<td>v122-02 progeny (47-169 x isolate 4)</td>
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</tr>
<tr>
<td>v122-08 progeny (47-169 x isolate 4)</td>
<td>47</td>
</tr>
<tr>
<td>v122-12 progeny (47-169 x isolate 4)</td>
<td>47</td>
</tr>
<tr>
<td>v122-13 progeny (47-169 x isolate 4)</td>
<td>47</td>
</tr>
<tr>
<td>v122-17 progeny (47-169 x isolate 4)</td>
<td>47</td>
</tr>
<tr>
<td>v122-18 progeny (47-169 x isolate 4)</td>
<td>47</td>
</tr>
</tbody>
</table>

### Table 7. MICs of recombinants with either the G54E substitution in Cyp51A (positive control) or the P88L substitution in HapE.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Substitutions</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyp51A</td>
<td>HapE</td>
</tr>
<tr>
<td>Transformation recipient akuBKU80</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Positive control Cyp51A (1)</td>
<td>G54E</td>
<td>-</td>
</tr>
<tr>
<td>Positive control Cyp51A (2)</td>
<td>G54E</td>
<td>-</td>
</tr>
<tr>
<td>HapE (1)</td>
<td>-</td>
<td>P88L</td>
</tr>
<tr>
<td>HapE (2)</td>
<td>-</td>
<td>P88L</td>
</tr>
</tbody>
</table>

MIC, minimum inhibitory concentration; ITZ, itraconazole; VOR, voriconazole; POS, posaconazole.
Figure 2. *In vitro* growth curves of susceptible and resistant progeny.
A. *In vitro* growth curves of susceptible (*n*=12) and resistant (*n*=4) progeny.
B. The maximum growth per hour (6-10 hours, between dashed lines of Figure 2A) was averaged (±standard deviation) for susceptible and resistant progeny. The difference is significant according to a Student’s T-test (one-tailed, heteroscedastic).

Figure 3. *Cyp51A* mRNA levels in the parental isolates (47-169, isolate 3, isolate 4) and three of the progeny (v122-12, v121-64, v121-48).
Isolate v122-12 contains only the HapE substitution and not the other five gene mutations. As a control, we selected two other progeny: v121-64 containing only the erg25 mutation and v121-48 containing the erg6/erg25 double mutation. S: susceptible, R: resistant.
A hapE mutation that causes azole resistance

Transformations
Homologous gene replacement studies with the six mutated genes from isolate 3 were performed using cyp51A G54E as a positive control (Table 2). After selection on itraconazole-containing medium, successful recombinants were only obtained from the positive control cyp51A and from candidate resistance gene hapE (two recombinants each, Table 7). Subsequent sequencing of the complete cyp51A gene and promoter region and the hapE gene showed no additional mutations, indicating that only the P88L- (HapE) or the G54E- (Cyp51A) causing mutation was introduced by the homologous gene replacement. The positive control substitution G54E in Cyp51A is known to cause resistance to itraconazole and posaconazole [22,33,57-58], and indeed both recombinants showed resistance to itraconazole and posaconazole. Moreover, both HapE P88L recombinants showed resistance to itraconazole with an MIC of >16 mg/l, as was also observed in patient isolates 3 and 4 (cf. Figure 1 and Table 7). As observed in the patient isolates, the voriconazole and posaconazole MICs increased two dilution steps from 0.25 to 1 and from 0.032 to 0.125, respectively (cf. isolates 2 and 3 in Figure 1). These results show that the HapE P88L substitution is the only one of the six mutations in isolate 3 capable of conferring the azole resistant phenotype.

HapE sequencing in resistant isolates without cyp51A mutations
Our collection contained eleven resistant A. fumigatus isolates without cyp51A mutations known to cause resistance. Eight were clinical isolates and the remaining three isolates were of environmental origin. Although some of the isolates did show polymorphisms in intronic regions of the gene, we never observed the mutation resulting in P88L or any other mutation within the exonic regions.

Discussion
In our study the whole genome sequencing strategy was used to identify a resistance mutation that arose as a de novo mutation in a CGD patient with chronic fibrotic pulmonary aspergillosis caused by A. fumigatus. We selected two A. fumigatus isolates, taken just before and just after the transition from a susceptible to an azole-resistant phenotype. Although the resistant strain (isolate 3) was recovered only 17 weeks after the susceptible strain (isolate 2), we identified no less than six non-synonymous mutations. Moreover, if we use the validated product quality score as a reliable cutoff (3,000), we found 16 additional mutations, bringing the total number of mutations to at least 22 (Table S2, available at http://dx.plos.org/10.1371/journal.pone.0050034). These included five mutations in non-coding regions and 11 cases where the resistant strain was synonymous to the susceptible reference genomes A. fumigatus Af293 and A. fumigatus A1163 and thus unlikely to be responsible
for the resistant phenotype. This may indicate that *A. fumigatus* undergoes many genetic changes during human infection, possibly as a response to the stressful environment. Potentially, the resistant strain diverged from the dominant susceptible lineage even before the last susceptible strain was isolated. This minority strain may have been accumulating neutral or slightly deleterious mutations before finally the resistance causing mutation allowed it to dominate the *A. fumigatus* population in the CGD patient.

Because we identified more than one non-synonymous mutation, we chose sexual crossing as an additional selection method. After crossing the patient isolates with an azole susceptible isolate that did not contain any of the six mutations, the mutation in *hapE* was identified as the most likely cause of azole resistance as this was the only mutation present in all resistant progeny. Subsequent gene replacement experiments provided the final proof that the *HapE P88L* substitution was the cause of the resistance phenotype observed.

With respect to the sexual reproduction experiment, part of the progeny of isolates 3 and 4 exhibited trailing growth (partial inhibition of growth over an extended range of antifungal concentrations [59]) in the presence of itraconazole. However, these trailing isolates did show clear susceptible MIC endpoints for voriconazole and posaconazole. Trailing endpoints caused problems when testing *Candida* spp. with azoles as well, but animal models and clinical experience argue that trailing endpoints do not indicate true resistance [56,60-61], suggesting that the progeny with trailing endpoints for itraconazole might not be truly resistant.

Furthermore, we noted that after sexual reproduction, only 18-30% of the progeny contained the *hapE* mutation instead of the expected 50%. Using *in vitro* growth kinetics in fluid medium, it was previously shown that the resistant isolates 3 and 4 have a significantly reduced growth rate and especially delayed germination compared to susceptible isolates 1 and 2, possibly associated with the resistance mechanism [46]. Therefore we hypothesized that the lower than expected recovery rate of the *hapE* mutation in the progeny might be due to slow germination, as we isolated the germinated sexual spores based on their growth on the medium and not by randomly selecting ascospores by using the microscope. Possibly only the faster germinating spores were collected resulting in a lower percentage of the slower germinating, resistant (*hapE* mutated) progeny. Determination of the *in vitro* growth curves confirmed a significant decrease in growth rate of azole resistant compared to azole susceptible progeny (Figure 2 and Figure S1, available at http://dx.plos.org/10.1371/journal.pone.0050034). Furthermore, we could speculate that the resistance mechanism is indeed associated with loss of fitness (alteration of growth and virulence) observed in the resistant isolates, as suggested before [46]. We presume that the resistant isolates described here are disadvantaged in the absence of azoles as a consequence of the *hapE* mutation. In contrast, for isolates with mutations in *cyp51A*, there is no evidence of fitness loss *in vivo* [Mavridou E, Meletiadis J, Arendrup MC, Melchers WJ, Mouton JW, Verweij PE. Impact of CYP51A
A *hapE* mutation that causes azole resistance

mutations associated with azole-resistance on *in vitro* growth rates and *in vivo* virulence of clinical *Aspergillus fumigatus* isolates. 20th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Vienna, April 2010; abstract O345).

Remarkably, two of the five other validated mutations were situated in genes involved in the ergosterol biosynthesis pathway (*i.e.* erg6 and erg25). *A. fumigatus* contains two potentially redundant copies of these genes (erg6: AFUA_4G03630/AFUB_099400 and AFUA_4G09190/AFUB_066290; erg25: AFUA_4G04820/AFUB_098170 and AFUA_8G02440/AFUB_084150; as determined by the CADRE genome browser [62,63] and literature [64,65]). Both genes function downstream of the cyp51 (erg11) gene, at least in *Saccharomyces cerevisiae* [66]. It has been shown that downstream genes erg3 [29,31,37,67], erg6, and erg28 [67] are involved in resistance in other fungi. In addition, erg6 and erg25 are both up-regulated in amphotericin B / fluconazole resistant *C. albicans*, suggesting a possible role of these two genes in acquired resistance [68]. Neither erg6 nor erg25 have ever been described to impact on azole susceptibility in *A. fumigatus*, but many erg genes, amongst which erg25, have been shown to be differentially expressed after voriconazole exposure [69]. Nevertheless, we found no evidence that the mutations in either erg6 or erg25 are associated with the resistant phenotype in isolates 3 and 4; neither from the sexual crossing- nor from the transformation experiments. In addition, the erg6/erg25 double mutation was found in azole susceptible progeny, excluding the possibility that both mutations together would result in the resistant phenotype (results not shown).

The *hapE* gene encodes the CCAAT-binding factor complex subunit HapE. Besides HapE, the CCAAT-binding complex (Hap-complex) consists of at least two other subunits: HapB and HapC. The complex is a transcription factor that specifically recognizes the regulatory CCAAT element found in the forward or reverse orientation in promoter regions of numerous eukaryotic genes [70]. The *hapE* gene contains four exons and the mutation is situated in the fourth exon, in the evolutionary conserved core domain [62,63,71], suggesting that P88 is an important amino acid for the function of the protein. Moreover, in the human NF-YC (the mammalian HapE homologue) deletion of residues 43-45 (homologous to *A. fumigatus* residues 87-89), are deleterious for DNA binding [72], indicating that P88 is needed for the Hap-complex to bind the regulatory CCAAT element and initiate transcription.

Initially, we hypothesized that the resistance observed in the HapE-mutant might be HapX-mediated. HapX has been shown to physically interact with the Hap-complex (as shown in *A. nidulans* [73]) and is important in adaptation to conditions of iron starvation. Conversely, in conditions of iron sufficiency, HapX expression is repressed [65]. HapX deficiency causes significant attenuation of virulence in a murine model of aspergillosis [65]. As our resistant patient isolates also showed reduced virulence [46], we tested the isolates for their azole susceptibility in the presence and absence of iron to investigate whether the resistance (and attenuated virulence) observed could be HapX-mediated. However, our results indicated that
the presence of iron did not have any effect on susceptibility (results not shown), suggesting that resistance may not be mediated by HapX.

We have previously observed that the cyp51A expression level of isolates 3 and 4 was four to six-fold higher compared to isolates 1 and 2 [46]. In addition, analysis of the sexual progeny showed that the hapE mutant has an increased cyp51A mRNA expression compared to progeny without the hapE mutation. As Hap is a transcription factor complex, the increased resistance might be due to a gain of function mutation if the mutated Hap-complex binds to a CCAAT-box in the promoter region of cyp51A and induces the expression of the gene. Alternatively, Hap might function as a transcriptional repressor, and the hapE P88L substitution could de-repress cyp51A expression. A CCAAT-box is present in the 5′ upstream region of A. fumigatus cyp51A, located at -197 bp from the start codon, though additional analyses are needed to confirm the binding of the (mutated) Hap-complex. Gain-of-function mutations in transcription factors have already been shown to be linked to increased antifungal drug resistance in C. albicans [74-79].

The NF-Y (the mammalian Hap-complex)/SREBP (sterol-regulatory element binding protein) combination regulates essentially all genes involved in cholesterol metabolism [80], indicating that the fungal ergosterol biosynthesis (the pathway analogous to the mammalian cholesterol biosynthesis), may also be mediated by these regulators. An in vitro null-mutant of the A. fumigatus SREBP transcription factor SrbA has recently been shown to be highly susceptible to fluconazole and voriconazole [45]. A subsequent study suggested that the increased fluconazole susceptibility in the absence of SrbA was partially due to loss of iron homeostasis [81]. Furthermore, SrbA is a direct transcriptional regulator of cyp51A, being the likely underlying mechanism of the increased susceptibility of the deletion mutant [82]. It currently remains unclear whether the declined expression is indeed the cause of the increasedazole susceptibility and there has been no evidence for involvement of SrbA in resistance in clinical cases. However, these data indicate that alterations in SREBP transcription factors such as SrbA or NF-Y (Hap) transcription factor subunits such as HapE may indeed initiate changes altering cyp51A expression and subsequently lead to azole resistance.

In conclusion, by combining a comparative genomic and genetic approach, we have discovered a novel triazole resistance mutation in A. fumigatus caused by a single base substitution in the transcription factor subunit HapE. We were fortunate to have a set of isogenic strains, i.e. isolates with a similar genetic background, and a comparison of the whole genome sequences of isolates 2 and 3 shortlisted 69 potential non-synonymous mutations. Through resequencing of candidate mutations, and the addition of sexual crosses, we were able to significantly reduce the selection of candidate mutations for the azole resistant phenotype. Over the last decade, an increase of azole resistance in A. fumigatus isolates has been observed [9-12]. Importantly, patients suffering from azole-resistant Aspergillus disease may
fail to respond to therapy [11,13-19]. Insight in the mutations that cause resistance can help to understand the epidemiology, find new targets for antifungal compounds and to develop diagnostic tools. The need to identify resistance mechanisms was underscored in a recent paper estimating that in 54% of patients with an azole resistant \textit{A. fumigatus} isolate the resistance mechanism was not Cyp51A-mediated and thus remained unexplained [9]. This is a challenge for the early diagnosis of azole-resistance in patients with \textit{Aspergillus} diseases. In these patients fungal cultures often remain negative, which precludes \textit{in vitro} susceptibility testing. Furthermore, a recent study showed that the use of molecular tools for detection of \textit{cyp51A}-mutations directly in clinical specimens was much more sensitive than culture [83]. However, knowledge of underlying resistance mechanisms is required in order to apply molecular techniques in patient care. The strategy that we have described will help to identify resistance mechanisms and improve our diagnostic tools in order to ultimately improve the outcome of patients with azole resistant \textit{Aspergillus} diseases.

**Acknowledgements**

The authors wish to thank C.M. O’Gorman and P.S. Dyer (School of Biology, University of Nottingham, Nottingham, UK) for providing \textit{A. fumigatus} isolate 47-169, used for sexual crossing experiments.
References

A hapE mutation that causes azole resistance


A hapE mutation that causes azole resistance


A hapE mutation that causes azole resistance


A *hapE* mutation that causes azole resistance
A. fumigatus cleistothecia developed after mating on Oatmeal agar (picture: Hein van der Lee and Simone Camps).
Section II

Emergence of resistance through environmental exposure
Agarose gel electrophoresis image of *A. fumigatus cyp51A* promotor region amplicons. Lane 1 and 7, molecular weight marker; lane 2, wildtype promotor; lane 3, TR$^{34}$; lane 4, TR$^{46}$; lane 5, TR$^{53}$; lane 6, triplicate of the 34bp sequence induced after passage of *A. fumigatus TR*$_{34}$ on tebuconazole.
Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*

Snelders E*, Camps SMT*, Karawajczyk A, Schaftenaar G, Kema GHJ, van der Lee HAL, Klaassen CHW, Melchers WJG, Verweij PE


*contributed equally to this publication
Azoles play an important role in the management of Aspergillus diseases. Azole resistance is an emerging global problem in Aspergillus fumigatus, and may develop through patient therapy. In addition, an environmental route of resistance development has been suggested through exposure to 14α-demethylase inhibitors (DMIs). The main resistance mechanism associated with this putative fungicide-driven route is a combination of alterations in the cyp51A gene (TR\textsubscript{34}/L98H). We investigated if TR\textsubscript{34}/L98H could have developed through exposure to DMIs. Thirty-one compounds that have been authorized for use as fungicides, herbicides, herbicide safeners and plant growth regulators in the Netherlands between 1970 and 2005, were investigated for cross-resistance to medical triazoles. Furthermore, Cyp51-protein homology modeling and molecule alignment studies were performed to identify similarity in molecule structure and docking modes. Five triazole DMIs, propiconazole, bromuconazole, tebuconazole, epoxiconazole and difenoconazole, showed very similar molecule structures to the medical triazoles and adopted similar poses while docking the protein. These DMIs also showed the greatest cross-resistance and, importantly, were authorized for use between 1990 and 1996, directly preceding the recovery of the first clinical TR\textsubscript{34}/L98H isolate in 1998. Through microsatellite genotyping of TR\textsubscript{34}/L98H isolates we were able to calculate that the first isolate would have arisen in 1997, confirming the results of the abovementioned experiments. Finally, we performed induction experiments to investigate if TR\textsubscript{34}/L98H could be induced under laboratory conditions. One isolate evolved from two copies of the tandem repeat to three, indicating that fungicide pressure can indeed result in these genomic changes. Our findings support a fungicide-driven route of TR\textsubscript{34}/L98H development in A. fumigatus. Similar molecule structure characteristics of five triazole DMIs and the three medical triazoles appear the underlying mechanism of cross resistance development. Our findings have major implications for the assessment of health risks associated with the use of triazole DMIs.
Introduction

Aspergillus fumigatus is the most frequent cause of Aspergillus diseases in humans, which include allergic syndromes, aspergilloma and chronic or acute invasive aspergillosis. Antifungal agents of the azole class play a prominent role in the management of Aspergillus diseases. Three medical triazoles, itraconazole, voriconazole and posaconazole, are clinically licensed for the prevention and treatment of Aspergillus diseases [1]. It has become apparent that A. fumigatus can develop resistance to the medical triazoles. Azole resistance is commonly due to mutations in the cyp51A-gene, encoding the target enzyme of antifungal azoles, and both preclinical evidence and clinical experience suggests that reduced in vitro susceptibility is associated with increased probability of failure to azole therapy [2-4]. Azole resistance may develop during azole therapy, which has been primarily reported in patients with aspergilloma or other Aspergillus cavities that received long-term azole therapy [5]. This route of resistance development is characterized by recovery of azole-resistant A. fumigatus isolates exclusively from patients receiving azole therapy and by a high diversity of resistance mechanisms. Sometimes multiple resistance mechanisms were found in different A. fumigatus colonies recovered from a single patient [5]. The morphotype of A. fumigatus appears to be important as the fungus commonly sporulates in cavities, thus creating spores that harbor cyp51A-mutations [6].

In the Netherlands, we observed azole resistance in A. fumigatus isolates from azole-naïve patients and in patients with invasive aspergillosis, which is characterized by hyphal growth in the absence of asexual reproduction. One explanation for this observation could be that a second route of resistance development may exist through environmental exposure of A. fumigatus to 14α-demethylase inhibitors (DMIs) [4, 6-7]. DMIs are abundantly used for crop protection against phytopathogenic molds, for prevention of spoilage post harvest and for preservation of materials. Evidence that supports such a route of resistance development is the dominance of a single resistance mechanism in over 90% of Dutch azole-resistant A. fumigatus isolates, recovered from epidemiologically unrelated patients [6-8]. This mechanism consists of a 34-bp insertion in the promoter region of the cyp51A gene combined with a substitution at codon 98 of leucine to histidine (TR$_{34}$/L98H) [9]. TR$_{34}$/L98H isolates were cultured from patients that were azole-naïve as well as those with previous azole exposure, and were also recovered from the environment [10]. Genetic analysis showed clustering of clinical and environmental TR$_{34}$/L98H isolates compared to wild type controls [7,10]. TR$_{34}$/L98H isolates exhibit a multi-azole-resistant phenotype and azole-resistant invasive aspergillosis was associated with a high mortality rate of 88% [8,11]. In the Netherlands the first TR$_{34}$/L98H isolate was cultured in 1998 and since then the prevalence of clinical isolates harboring TR$_{34}$/L98H has increased over time [7-8].
Chapter 4

Theoretically there are significant risks associated with the environmental route of resistance development in fungi. First there is the potential of migration of the resistance trait through sexual or asexual reproduction. It has been shown for phytopathogens that resistance mechanisms may develop locally and subsequently spread across countries [12]. There are early indications that suggest that migration is occurring in TR$_{34}$/L98H as isolates harboring this resistance mechanism have now been reported in other European countries [13], and more recently in azole-resistant isolates in China [14]. The other risk of the environmental route of resistance development is the emergence of multiple resistance mechanisms over time due to continuedazole pressure. There are recent reports that indicate that in addition to TR$_{34}$/L98H other ‘environmental’ resistance mechanisms may be emerging [15-16]. Therefore, it is of great importance to explore the relationship between the use of DMIs and the emergence of TR$_{34}$/L98H in A. fumigatus as this may enable effective measures to be taken that prevent further increase of TR$_{34}$/L98H isolates or of the emergence of new resistance mechanisms. The aim of our current research was to determine if the route of TR$_{34}$/L98H development could have been fungicide driven. Our hypothesis was that cross-resistance could develop if DMIs and medical triazoles share similar molecule characteristics. This was investigated through molecule alignment and docking studies using a homology model of the Cyp51A protein. Furthermore, temporal relationships between DMI exposure and TR$_{34}$/L98H emergence were investigated. Finally, we investigated if the TR$_{34}$/L98H substitutions could be induced through DMI-exposure under laboratory conditions. We were able to identify five triazole DMIs that exhibit highly similar molecule characteristics to medical triazoles and could have caused the emergence of TR$_{34}$/L98H in A. fumigatus.

**Materials and methods**

**Susceptibility testing**

A collection of 25 clinical wild type A. fumigatus isolates, 25 clinical azole-resistant TR$_{34}$/L98H isolates, 17 environmental wild type isolates, and 13 environmental TR$_{34}$/L98H isolates were selected for investigation of the *in vitro* activity of fungicides. In addition, two clinical isolates were included that have a tandem repeat as underlying resistance mechanism similar to TR$_{34}$/L98H: one isolate harbored a 53-bp tandem repeat and the other a 46-bp tandem repeat in combination with two substitutions in the *cyp51A*-gene at codons 121 and 289. Finally, four isolates were included that had a resistance mechanism that arose through azole therapy, consisting of point mutations in the *cyp51A*-gene. All isolates were previously identified by sequencing parts of the β-tubulin gene and the calmodulin gene. Furthermore, the full coding sequence and promoter region of the *cyp51A*-gene was sequenced and microsatellite genotyping was performed [7]. Sequences were aligned with a reference *cyp51A* sequence (GenBank accession no. AF338659) to identify mutations. All isolates were stored in 10%
Triazole fungicides can cause cross-resistance to medical triazoles

glycerol at −80°C and subcultured on Sabauroud slants at 37°C.
Between 1970 and 2005 33 compounds were authorized by the Dutch Board for the
Authorization of Plant Protection Products and Biocides for use as fungicides, herbicides,
herbicide safeners and plant growth regulators, in The Netherlands. Of these 31 were available
for testing including: amitrole, benomyl, bitranol, bromuconazole, carbendazim, cyazofamid,
cyproconazole, difenoconazole, epoxiconazole, fenamidone, fenarimol, fenchlorazole-ethyl,
fuberidazole, imazamethabenz-methyl, imazili, metconazole, myclobutanil, nuarimol,
paclobutrazole, penconazole, prochloraz, propiconazole, prothioconazole, pyrimethanil,
tebuconazole, thiabendazole, thiophanate-methyl, triadimefon, triadimenol I, triadimenol II,
triflumizole (Sigma Aldrich). The compounds were dissolved in DMSO and autosterilized for 30
minutes at room temperature. The minimal inhibiting concentration (MIC) was determined
using a microbroth dilution format according to the CLSI M38-A2 reference method [17].

Docking studies
The structure of wild type Cyp51A protein *A. fumigatus* was derived from the crystal
structure of human (PDB code: 3I3K) and *Mycobacterium tuberculosis* (Mt) (PDB code:
1EA1) lanosterol 14α-demethylase by homology modeling. Both proteins share 38% and
24% sequence identity with Cyp51A of *A. fumigatus*, respectively and contain ligands in
the active site bound to heme. The three-dimensional structures have been predicted by
YASARA’s homology modeling experiment (http://www.yasara.org). The experiment consists
from building four models based on different alignment variants. The missing loops were
modeled and optimizations of structures were performed. The model with the best Z-score
derived from the crystal structure of human lanosterol 14α-demethylase was used for the
presented studies.

In a recent publication by Fraczek et al. the *Mycobacterium* and human structures were
also both compared and confirmed the choice for the human lanosterol 14α-demethylase
as the best template for the *A. fumigatus* model [18]. The structures of tested fungicides
and medical triazoles (Table 1) were downloaded from PubChem (http://pubchem.ncbi.nlm.
nih.gov/). We used FlexX for the docking experiment [19-20]. The coordination of ligands
to the iron atom of heme was treated as pharmacophore during the docking procedure.
The water molecule present in the active site according to the crystal structure of 1EA1 was
treated dynamically. The program checked automatically whether the presence of the water
molecule had favorable contribution to the docking pose and only in such a case the water
molecule was reported back, otherwise it was neglected. The flexibility of hydrogen atoms of
Y107, Y121 and S297 was introduced to find an optimal docking pose for the ligand. Docking
the respective compounds back into their crystal structure validated the docking procedure.
The root mean square deviation (RMSD) of the positions for fluconazole (PDB code 1EA1) was
0.28 Å and for ketoconazole (PDB code 313K) 0.44 Å. All the binding modes present in the
crystal structures were conserved [21-22].
<table>
<thead>
<tr>
<th>Compound</th>
<th>Target site of action</th>
<th>Chemical group</th>
<th>Year</th>
<th>Clinical wild type</th>
<th>Environment wild type</th>
<th>Clinical TR/L98H</th>
<th>Environment TR/L98H</th>
<th>Effect size r</th>
</tr>
</thead>
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<td>Itraconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1991</td>
<td>0.125 (0.063–0.5)</td>
<td>0.25 (0.125–1)</td>
<td>32 (4–32)</td>
<td>32 (16–32)</td>
<td>0.99</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>2001</td>
<td>0.5 (0.5–2)</td>
<td>0.5 (0.5–4)</td>
<td>4 (2–8)</td>
<td>4 (1–32)</td>
<td>0.82</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>2006</td>
<td>0.031 (0.016–1)</td>
<td>0.063 (0.031–0.25)</td>
<td>0.5 (0.25–1)</td>
<td>0.5 (0.25–0.5)</td>
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<tr>
<td>Carbendazim</td>
<td>Methyl Benimidazole Carbamates</td>
<td>benimidazoles</td>
<td>1973</td>
<td>2 (1–16)</td>
<td>8 (2–16)</td>
<td>4 (1–32)</td>
<td>4 (1–32)</td>
<td>0.17</td>
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<tr>
<td>Fuberidazole</td>
<td>Methyl Benimidazole Carbamates</td>
<td>benimidazoles</td>
<td>1973</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>0*</td>
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<tr>
<td>Thiabendazole</td>
<td>Methyl Benimidazole Carbamates</td>
<td>benimidazoles</td>
<td>1973</td>
<td>32 (32)</td>
<td>32 (16–32)</td>
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<td>32 (32)</td>
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<td>Benomyl</td>
<td>Methyl Benimidazole Carbamates</td>
<td>benimidazoles</td>
<td>1975</td>
<td>2 (2–8)</td>
<td>4 (2–16)</td>
<td>8 (2–32)</td>
<td>4 (2–32)</td>
<td>0.31</td>
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<td>Thiophanate-methyl</td>
<td>Methyl Benimidazole Carbamates</td>
<td>thiophanates</td>
<td>1975</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (16–32)</td>
<td>0.01</td>
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<tr>
<td>Cyazofamid</td>
<td>Quinone inside Inhibitors cyanimidazoles</td>
<td>2002</td>
<td>32 (32)</td>
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<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>0*</td>
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<tr>
<td>Imazalil</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>imidazoles</td>
<td>1978</td>
<td>0.125 (0.125–0.5)</td>
<td>0.25 (0.125–0.5)</td>
<td>2 (1–8)</td>
<td>2 (2–8)</td>
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<td>Prochloraz</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>imidazoles</td>
<td>1987</td>
<td>0.5 (0.25–0.5)</td>
<td>0.5 (0.125–0.5)</td>
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<td>1 (1–32)</td>
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<td>imidazoles</td>
<td>1992</td>
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<td>8 (4–32)</td>
<td>32 (8–32)</td>
<td>32 (32)</td>
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<td>Imazethabenz methyl</td>
<td>Aceetoxyacid synthase inhibitors</td>
<td>imidazolinone</td>
<td>1993</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (16–32)</td>
<td>32 (32)</td>
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<td>Fenarimol</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>pyrimidines</td>
<td>1983</td>
<td>8 (8–32)</td>
<td>8 (8–32)</td>
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<td>Nuarimol</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>pyrimidines</td>
<td>1993</td>
<td>16 (8–32)</td>
<td>16 (8–32)</td>
<td>32 (16–32)</td>
<td>32 (32)</td>
<td>0.84</td>
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<tr>
<td>Pyrimethanil</td>
<td>Methionine synthesis inhibitors</td>
<td>anilinopyrimidines</td>
<td>1995</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>0*</td>
</tr>
</tbody>
</table>

Table 1. Antifungal susceptibilities of medical triazoles and compounds used as fungicide, herbicide, herbicide safener and plant growth regulator.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Mode of Action</th>
<th>Imidazolinones</th>
<th>2005</th>
<th>32 (32)</th>
<th>32 (32)</th>
<th>32 (32)</th>
<th>32 (16–32)</th>
<th>32 (32)</th>
<th>0.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenamidone</td>
<td>Acetyl-CoA Carboxylase inhibitors</td>
<td>triazoles</td>
<td>1992</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>0*</td>
</tr>
<tr>
<td>Fenchlorazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1970</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>0*</td>
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<tr>
<td>Amitrole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1980</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>0*</td>
</tr>
<tr>
<td>Triadimefon</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1983</td>
<td>4 (2–32)</td>
<td>16 (2–32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>0.71</td>
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<td>Bitertanol</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1986</td>
<td>32 (16–32)</td>
<td>32 (16–32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
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<td>Penconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1988</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>0*</td>
</tr>
<tr>
<td>Triadimenol I</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1988</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>0*</td>
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<tr>
<td>Triadimenol II</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1990</td>
<td>2 (2–4)</td>
<td>2 (2–8)</td>
<td>32 (16–32)</td>
<td>32 (16–32)</td>
<td>0.96</td>
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<td>Propiconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1992</td>
<td>32 (8–32)</td>
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<td>Cyproconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1992</td>
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<td>16 (8–16)</td>
<td>0.93</td>
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<tr>
<td>Tebuconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1993</td>
<td>16 (8–32)</td>
<td>16 (4–32)</td>
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<td>Myclobutanil</td>
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<td>triazoles</td>
<td>1994</td>
<td>1 (1–2)</td>
<td>1 (1–4)</td>
<td>16 (8–32)</td>
<td>16 (8–16)</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>Difenoconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1994</td>
<td>2 (2–8)</td>
<td>2 (2–16)</td>
<td>32 (32)</td>
<td>32 (32)</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>Epoxiconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1996</td>
<td>1 (1–4)</td>
<td>1 (1–4)</td>
<td>16 (8–32)</td>
<td>16 (16–32)</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>Bromuconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>1997</td>
<td>16 (8–32)</td>
<td>16 (8–32)</td>
<td>32 (16–32)</td>
<td>32 (32)</td>
<td>0.82</td>
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<tr>
<td>Paclobutrazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>2005</td>
<td>0.25 (0.125–0.5)</td>
<td>0.25 (0.125–0.5)</td>
<td>2 (1–4)</td>
<td>1 (1–2)</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>Metconazole</td>
<td>DeMethylation Inhibitors SBI: Class I</td>
<td>triazoles</td>
<td>2005</td>
<td>8 (2–16)</td>
<td>8 (2–16)</td>
<td>16 (8–32)</td>
<td>16 (16–32)</td>
<td>0.71</td>
<td></td>
</tr>
</tbody>
</table>
*Cannot be computed because at least one of the variables is constant.
Microsatellite genotyping

Microsatellite genotyping was used to determine the genetic distances between TR$_{34}$/L98H A. fumigatus isolates. A collection of 144 consecutive TR$_{34}$/L98H isolates were used that originated from two prospective surveillance studies that were performed in the Netherlands. The first study included A. fumigatus isolates that were collected in Dutch hospitals between 1994 and 2007. A total of 1,912 A. fumigatus isolates were obtained from 1,219 patients from the Radboud University Nijmegen Medical Center [7]. In addition, 147 A. fumigatus isolates from 101 patients, from 28 other medical centers in the Netherlands were collected [4,7]. The second culture collection included 1,792 A. fumigatus isolates that were collected from 1,192 patients in seven University Medical Centers in the Netherlands between 2007 and 2009 [8]. Both studies included an unselected collection of A. fumigatus isolates (clinically relevant as well as colonizing isolates) and used agar supplemented with itraconazole to detect for azole-resistance.

From six loci, consisting of three tri- and three tetranucleotide repeats, fragments were amplified by using fluorescently labeled primers. The sizes of the fragments were determined by addition of the GeneScan LIZ[500] marker and subsequent analysis of the fragments on the Applied Biosystems 3730 DNA analyzer. Assignment of repeat numbers in each marker was determined from the GeneScan data by using the Peak Scanner version 1.0 software (Applied Biosystems) [23]. By plotting the number of observed new genotypes versus the time on a semi-logarithmic scale, the year that the first new genotype emerged in The Netherlands was calculated with a 95% confidence interval by using the software package GraphPad Prism v5.00.

Induction experiments

Induction experiments were performed with the medical triazole itraconazole (5 mg/l), the triazole DMIs bromuconazole (8 mg/l), difenoconazole (8 mg/l), epoxiconazole (16 and 32 mg/l), propiconazole (32 mg/l) and tebuconazole (8 mg/l) and all five DMIs combined together in concentrations ranging between 0.063-4 mg/l of each DMI. Wild type isolate CM237 and akuB$^{ku80}$ as well as recombinant A. fumigatus isolates akuB$^{ku80}$-TR3 containing the 34-bp tandem repeat and akuB$^{ku80}$-L98H2 containing the L98H substitution were used. A solution of $1\times10^6$ of conidia was spread on a GYEP agar plate (glucose 2%, yeast extract 0,3%, peptone 1% and agar 2%) containing one or a combination of DMIs and subsequently passaged on GYEP agar slants with the same concentration of DMI(s). Agar plates and slants were incubated at 37°C, and isolate akuB$^{ku80}$-TR3 was also incubated at 25°C and 48°C. After 10 passages sequencing of the cyp51A promoter and full coding gene was performed to detect mutations.
**Statistical analysis**

In order to express differences in MIC\(_{50}\) between wild type and TR\(_{34}/L98H\) for the different compounds we first log transformed the MIC\(_{50}\) data and then computed point biserial correlations as correlation effect sizes (r) [24]. Values of r = 0 indicate similarity between MIC\(_{50}\)’s and values of r = 1 indicate the largest relative dissimilarity. These correlation effect sizes cannot be computed in cases where all samples have identical MIC\(_{50}\) values, such as with compounds that show no in vitro activity against both wild type and TR\(_{34}/L98H\) isolates. In those cases the correlation effect size was considered r = 0.

**Results**

**Activity of fungicides against A. fumigatus**

In the Netherlands 33 compounds have been authorized by the Dutch Board for the Authorization of Plant Protection Products and Biocides for use as fungicides, herbicides, herbicide safeners and plant growth regulators, between 1970 and 2005, of these 19 were DMIs (Table 1; Figure 1; Figure 2A). We were able to obtain 31 of these compounds as dry powder and investigated the in vitro activity against 38 TR\(_{34}/L98H\) A. fumigatus isolates from clinical and environmental origin and 42 wild type controls. In addition, two azole-resistant isolates from environmental origin that harbor a transcriptional enhancer as a resistance mechanism and four isolates with point mutations in the cyp51A-gene that arose through patient therapy were also tested (Table 2). Differences in MIC\(_{50}\) between the wild type and TR\(_{34}/L98H\) against all different compounds were computed as correlation effect sizes (r). The correlation coefficient is used as a measure of the size of an effect with a value of −1 indicating a negative correlation between the two variables, a value of 0 indicating no correlation and a value of 1 indicating a positive correlation. For the medical triazoles the effect sizes were 0.99 for itraconazole, 0.82 for voriconazole and 0.85 for posaconazole representing a positive correlation of dissimilarity between the MIC\(_{50}\)’s of the wild type and TR\(_{34}/L98H\) isolates. Dissimilarity between the MIC\(_{50}\)’s was found for 20 compounds, with the greatest differences (r>0.90) found for propiconazole, difenoconazole, epoxiconazole (r = 0.96), bromuconazole (r = 0.95), metconazole (r = 0.94), imazalil (r = 0.94), and tebuconazole (r = 0.93) (Figure 2B). These compounds were DMIs from the triazole class, with the exception of imazalil. Isolates with a 46 bp or 53 bp tandem repeat insertion showed similar correlation effect sizes as TR\(_{34}/L98H\) isolates (data not shown), while isolates that had become resistant through patient azole therapy generally showed lower r-values (Table 1) [25].
Chapter 4

**Molecule alignments and docking**

We used a homology model of the *A. fumigatus* CYP-protein to predict the preferred orientation of DMI-compounds to form a stable complex with the 14α-lanosterol demethylase enzyme. A crystal structure of the *A. fumigatus* Cyp51A protein is not available, therefore to see structural similarities in Cyp51s for azole inhibition we superimposed the fluconazole-bound *Mycobacterium tuberculosis* (Mt) structure (PDB code 1EA1), the ketoconazole-bound human structure (PDB code 313K) and ketoconazole-bound *A. fumigatus* homology structure. Both fluconazole and ketoconazole bind to the heme iron via the nitrogen of an azole ring. The dihalogenated phenyl group, a common structural moiety of ketoconazole and fluconazole, occupied the same spaces at the active site of the heme molecule but interacts with the binding pockets lined by different residues when the human Cyp51, the Mt Cyp51 and the *A. fumigatus* Cyp51 homology model are aligned. In human and *A. fumigatus* Cyp51, residues Y145 and Y121, respectively formed van der Waals contacts with the dichlorophenyl group of ketoconazole, whereas their side-chain hydroxyl group made hydrogen bonds to the D-ring propionate ($\text{C}_2\text{H}_5\text{COO}^-$) of the heme. Residue Y131 (PDB code 313K) that is located in the B′ helix of the homology structure (Y107) is invariant in the Cyp51 family and involved in hydrogen-bond formation with heme A-ring propionate in all three structures. In the Mt structure, Y145-corresponding F89 is away from the active site due to the conformational flexibility of the B–C–helix region. Instead, R95 and R96 of Mt Cyp51 are near the heme and fluconazole difluorophenyl group (Figure 3). Thus, ketoconazole could bind to Mt enzyme, utilizing the same space as fluconazole for the dihalogenated phenyl ring, while the remainder of ketoconazole would occupy the access channel observed in the human enzyme although the channel would have to be open by relocation of some of the side chains like F78 and M433 (grey structure Figure 3). In the Mt structure, the hydroxyl group of fluconazole made a water-mediated hydrogen bond to the heme A-ring propionate. This water molecule is not observed in the human structure because the cycle ether group of ketoconazole filled in the space of water. In addition, in Mt and *A. fumigatus* Cyp51s an invariant H259/H296 residue from helix I is pointed into the active site, whereas the confirmation of the corresponding H314 in human Cyp51 prevents its interaction with the inhibitors. The itraconazole, posaconazole and voriconazole molecules were docked into the *A. fumigatus* homology structure. They showed the same binding pattern as described for the crystal structures and were able to align to the presented poses of fluconazole (representative of voriconazole) and ketoconazole (representative of itraconazole and posaconazole). The compounds from the groups of imidazoles, pyrimidines and triazoles adopted similar poses upon docking in the active site of the *A. fumigatus* as those observed for the medical triazoles. The largest dissimilarities were in the cases of compounds that lack a phenyl group next to the 5- or 6-member aromatic ring that coordinates to the iron center.
Triazole fungicides can cause cross-resistance to medical triazoles

Figure 1. Chemical structures of antifungal compounds.
Three medical antifungal compounds and 31 compounds that were authorized by the Dutch Board for the Authorization of Plant Protection Products and Biocides for use as fungicides, herbicides, herbicide safeners and plant growth regulators. The compounds are presented according to structural group.
Triazole fungicides can cause cross-resistance to medical triazoles

Previous page, Figure 2. Overview of introduction of the 31 compounds by year and correlation effect sizes.

A) Overview of compounds by year of authorization by the Dutch Board for the Authorization of Plant Protection Products and Biocides (data from the Dutch Foundation for Phytofarmacy, Nefyto). The five triazole DMIs that exhibited the most identical docking by molecule alignment are underlined in blue.

B) Correlation effect sizes (r) of compounds and medical triazoles comparing differences in the median MIC of wild type and TR$_{34}$/L98H isolates. The fungicides are represented by grey dots and those belonging to the DMIs by black. The medical triazoles are indicated in red, and the five triazole DMIs that exhibited the most identical docking by molecule alignment are indicated in blue.

*Correlation effect sizes could not be computed if in at least one of the two groups all variables were constant. This was the case with compounds that showed no in vitro activity against both wild type and TR$_{34}$/L98H A. fumigatus isolates, and the correlation effect size was considered 0.

Table 2. Activity of medical triazoles and five DMIs against clinical and environmental A. fumigatus isolates with different cyp51A-mediated resistance mechanisms.

<table>
<thead>
<tr>
<th>Resistance mechanism cyp51A gene*</th>
<th>Median MIC (mg/l)</th>
<th>Medical triazoles &amp; DMIs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Promoter region</td>
<td>Coding region</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>34 bp TR L98H</td>
<td>38</td>
<td>&gt;16</td>
</tr>
<tr>
<td>46 bp TR Y121F, T289A</td>
<td>2</td>
<td>&gt;16</td>
</tr>
<tr>
<td>53 bp TR –</td>
<td>1</td>
<td>&gt;16</td>
</tr>
<tr>
<td></td>
<td>–</td>
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<td></td>
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<td>–</td>
<td>M220I</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>M220V</td>
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</table>

*TR, tandem repeat.

*ITC, itraconazole; VCZ, voriconazole; POS, posaconazole.
Figure 3. 3D representation of three aligned structures of Cyp51 with the ligands in their active site, constructed by using the Yasara software. In green human Cyp51 bound with ketoconazole from PDB: 3I3K; in gray Mt bound with fluconazole from PDB: 1EA1; in cyan *A. fumigatus* bound with ketoconazole from the homology model. The ligands are represented in balls and sticks, only the residues important for binding a particular ligand are depicted in the picture and represented in sticks. Numbering of the residues corresponds with their colors to the models.
Triazole fungicides can cause cross-resistance to medical triazoles

Figure 4. Two-dimensional structure of voriconazole with indicated pharmacophores that were used to align and filter the 31 compounds (Table 1).
The figure was constructed by using Marvin Sketcher from ChemAxon (www.chemaxon.com).

Figure 5. Analysis of fungicide binding modes compared to the medical triazoles.
A) Binding modes of propiconazole. This fungicide exhibits the most similar binding modes compared to the medical triazoles located in the active site of human and A. fumigatus Cyp51.
B) Binding modes of tebuconazole. This fungicide exhibits the most similar binding modes compared to the medical triazoles located in the active site of Mt Cyp51. The main difference between A and B is the interactions with residue H296 in the active site, which is lacking in A.
We performed a flexible alignment of the compounds on the structure of voriconazole in order to find the most similar compounds. The pharmacophores used as a filter for the alignment that consist of 5/6-member aromatic ring containing at least one nitrogen atom, a hydrogen-bond donor or acceptor and the aromatic functional group (Figure 4). The structures classified to groups of benzimidazoles, cyanimidazoles together with prochloraz, imazamethabenz from imidazolinone, and pyrimethanil from pyrimidines and fenamidone and amitrole from triazoles were filtered out from the set of the ligands as not similar to the voriconazole molecule. The remaining 20 of 31 structures were considered to be similar to the medical triazoles. We focused our analysis of the docking poses on the compounds that satisfied the given pharmacophores for alignment.

**Docking poses of fungicides similar to medical triazoles**

The triazole DMIs that have three nitrogen atoms in the aromatic ring coordinated to the iron atom of heme made a hydrogen bond contact to residue S297, present in the active site of the *A. fumigatus* Cyp51 homology model. Residue H296, also present in the active site, interacted with most of the fungicides with the exception of imazalil, triflumizole, fenarimol, nuarimol, penconazole, metconazole that instead interacted with a bridging water molecule. Propiconazole, myclobutanil, difenoconazole lack any interaction with residue H296 or a bridging water molecule. Most of the DMIs share the core structure with medical triazoles and due to this similarity they adopt much the same poses in the active site of *A. fumigatus* as the medical triazoles. Propiconazole and bromuconazole exhibit the most alike poses with the core structure being the most similar to itraconazole and posaconazole (Figure 5A). Tebuconazole and epoxiconazole also adopted the most alike poses being most similar to voriconazole, except they interacted with residue H296 in the active site (Figure 5B). The analysis of the top three poses proposed by the docking program showed that these compounds were able to adopt also poses where they interacted with a bridging water molecule instead of H296. This makes the binding modes of propiconazole, bromuconazole, tebuconazole and epoxiconazole most identical to those represented by the medical triazoles. Difenoconazole (Table 1) was different in structure from the rest of the cross-resistant DMIs. Instead of one aromatic ring (Figure 5A and and 4B) it has a biphenyl moiety, upon docking this part was placed into the access channel where the long tail of medical azoles is normally located.

The above mentioned five triazole DMIs were also among the compounds with the highest r-value and showed complete loss of *in vitro* activity against *A. fumigatus* isolates harboring TR$_{34}$/L98H (Figure 2B, Table 1). Moreover, these five DMIs were authorized for use in the Netherlands between 1990 and 1996 (Figure 2A), which preceded the first known isolation of a clinical TR$_{34}$/L98H isolate in 1998 [7]. Imazalil and metconazole also showed a high correlation effect size (Figure 2A), but, unlike the five abovementioned triazole DMIs, retained *in vitro*
activity against TR$_{34}$/L98H isolates (median MIC of 2 mg/l) (Table 1). Docking studies and molecule alignments showed that imazalil and metconazole were less similar to the medical triazoles and therefore less likely to have caused the emergence of TR$_{34}$/L98H in *A. fumigatus*.

**Microsatellite genotyping**

*A. fumigatus* isolates from two Dutch surveillance studies were used to investigate the evolution of TR$_{34}$/L98H genotypes over time [7-8]. The collections were obtained prospectively over a 16 year period and included 3,847 isolates from 2,512 patients. All isolates were screened for azole resistance by subculture on agar supplemented with itraconazole. The collections included 144 consecutive TR$_{34}$/L98H isolates which were genetically characterized by short tandem repeat genotyping [23]. By plotting the number of observed new genotypes versus time on a semi-logarithmic scale, we calculated a rate of change of 1.37±0.05 genotype-1.year$^{-1}$. Using the rate of change to calculate the year of first emergence of TR$_{34}$/L98H, indicated that TR$_{34}$/L98H had developed in the year 1997 (95% CI: 1993.7-1999.7) (Figure 6).

**Figure 6.** The evolution of new microsatellite genotypes over time based on short tandem repeat typing of 144 TR$_{34}$/L98H *A. fumigatus* isolates, cultured between 1998 and 2009 in the Netherlands. By plotting the number of observed new genotypes versus time on a semi-logarithmic scale, a rate of change of 1.37±0.05 genotype-1.year$^{-1}$ was calculated. As the first TR$_{34}$/L98H isolate was cultured in 1998, the rate of change indicates that the first strain would have emerged around 1997 (95% CI: 1993.7-1999.7). This analysis also indicates that TR$_{34}$/L98H had developed from a single ancestor.
Induction of TR\textsubscript{34}/L98H

We investigated if the TR\textsubscript{34}/L98H substitutions could be induced during exposure to DMIs under laboratory conditions. A wild type A. fumigatus isolate and recombinants containing either the 34-bp insertion or the L98H substitution were exposed to itraconazole, bromuconazole, difenoconazole, epoxiconazole, propiconazole, tebuconazole or a mixture of these DMIs. The induction experiments generally resulted in a resistant phenotype within three passages. In three out of twelve clones of A. fumigatus cultured under itraconazole pressure, cyp51A-substitutions G138C or P216L were detected. These substitutions have been reported in patients who developed azole-resistant Aspergillus disease during itraconazole therapy [5]. TR\textsubscript{34}/L98H was not found in any of the clones that were exposed to itraconazole, single DMI compounds or to a mixture of DMIs. However, following exposure of the A. fumigatus conidia containing the 34-bp insertion in the cyp51A-gene promoter to 8 mg/l of tebuconazole resulted in one clone in which after three passages a triplicate of the 34 bp sequence was detected in the promoter region.

Discussion

Although the hypothesis of a fungicide-driven route of azole resistance development in A. fumigatus is controversial [26], we provide evidence that such a route may exist. Five triazole DMIs were identified that exhibited very similar molecule characteristics to the medical triazoles, resulting in the most identical binding modes and the greatest level of cross-resistance. These five DMIs were authorized for use between 1990 and 1996, which was in keeping with our calculated date of origin of TR\textsubscript{34}/L98H based on microsatellite typing, and precedes the first clinical TR\textsubscript{34}/L98H isolate in 1998. Continued triazole DMI pressure and lack of an apparent fitness cost in TR\textsubscript{34}/L98H isolates are probably important factors that have facilitated the ability of TR\textsubscript{34}/L98H to sustain in the field in competition with wild type isolates. Although the relation between the use of antimicrobial agents outside human medicine and the development of resistance to clinically used compounds has been shown for bacteria, we show for the first time evidence that the same principle may occur in molds. Culture-based surveillance studies increasingly report TR\textsubscript{34}/L98H in clinical and environmental isolates in Europe and, most recently, in China [14]. Moreover, there is very recent evidence that two new ‘environmental’ azole resistance mechanisms have emerged in A. fumigatus in the Netherlands, of which one has rapidly migrated across the country similar to TR\textsubscript{34}/L98H [27]. However, surveillance studies based on positive cultures may underestimate the prevalence of resistance. Detection of azole resistance mechanisms directly in clinical specimens from patients with chronic lung diseases showed that in culture-negative, PCR-positive samples cyp51A-mutations were detectable in as many as 55.1% of respiratory samples [28]. These
Triazole fungicides can cause cross-resistance to medical triazoles

observations indicate that we are just beginning to understand the scale of the problem, but it suggests that azole resistance in *A. fumigatus* has become a public health problem and threatens an increasing population of (immunocompromised) patients. Our study was limited by the fact that we were unable to induce the full TR$_{34}/$L98H resistance mechanism during DMI-pressure under laboratory conditions, using an isolate that is deficient in DNA break repair. Previously, microsatellite genotyping showed shorter genetic distances for TR$_{34}/$L98H isolates compared with wild type isolates [10], which suggests that TR$_{34}/$L98H isolates may have originated from a common ancestor. If this would be the case, the development of TR$_{34}/$L98H would be extremely infrequent in the environment and would explain why we were unable to induce TR$_{34}/$L98H under laboratory conditions. However, this may point to other reasons for the emergence of TR$_{34}/$L98H. TR$_{34}/$L98H isolates may have other properties, such as increased fitness or virulence, or high sporulation efficacy, that have made isolates harboring TR$_{34}/$L98H more successful in the field than wild type *A. fumigatus*. However, at present there is no evidence that supports increased virulence in TR$_{34}/$L98H isolates. Animal studies indicate that the virulence of TR$_{34}/$L98H is similar to that of wild type isolates, although only one TR$_{34}/$L98H isolate was used [3]. An alternative explanation for our inability to induce TR$_{34}/$L98H could be that this resistance mechanism developed through sexual or parasexual reproduction rather than asexual reproduction, which was not tested in the laboratory. However, we did observe one isolate in which two copies of the tandem repeat evolved into three, supporting the role of DMIs in inducing genomic changes in cyp51A of *A. fumigatus*. Another limiting factor of our studies was the lack of sequence-based evolutionary analysis. In *A. fumigatus* no genes have been described that are suitable for this type of analysis and therefore we used microsatellite data.

The relation between the use of the triazole DMIs and cross-resistance to medical triazoles in *A. fumigatus* has major implications for the assessment of health risks associated with the use of DMIs. Molecule structure similarity and activity of triazole DMIs against *A. fumigatus* appear to be the key features that cause cross-resistance to medical triazoles. Further research should be aimed at understanding the conditions under which resistance mechanisms develop in the environment and which *Aspergillus* morphotype is most prone to develop resistance mechanisms. Reversal of resistance development may be achievable by restriction of certain triazole DMIs, but laboratory population studies and genetic mapping would be required to predict the impact of changes in DMI-pressure. In addition, there is limited insight in the use of fungicides for agricultural and non-agricultural applications. The continued use of DMIs with activity against opportunistic human fungal pathogens is a risk for the management of fungal diseases caused by these pathogens. The number of classes of drugs available for treating non-invasive and invasive fungal diseases is limited and the triazoles are the only class of antifungal agents that can be administered orally. A fungicide-driven route of resistance development in TR$_{34}/$L98H could indicate that such
mechanisms may also occur in other *Aspergillus* species or other opportunistic fungi. It is therefore of great importance to perform abovementioned research as it may allow the implementation of evidence-based strategies aimed at elimination of the fungicide-driven route of azole resistance development in opportunistic fungi.
Triazole fungicides can cause cross-resistance to medical triazoles

References


DNA chromatograms of *A. fumigatus* cyp51A surrounding codon 98. The top chromatogram represents the sequence derived from a susceptible isolate. The bottom chromatogram represents the sequence derived from a resistant isolate. Mutation T364A results into the Cyp51A L98H substitution.
Molecular epidemiology of *Aspergillus fumigatus* isolates harboring the TR$_{34}$/L98H azole resistance mechanism

Camps SMT, Rijs AJMM, Klaassen CHW, Meis JF, O’Gorman CM, Dyer PS, Melchers WJG, Verweij PE

Abstract

A rapid emergence of azole resistance has been observed in *Aspergillus fumigatus* in The Netherlands over the past decade. The dominant resistance mechanism appears to be of environmental origin and involves the TR$_{34}$/L98H mutations in *cyp51A*. This resistance mechanism is now also increasingly being found in other countries. Therefore, genetic markers were used to gain more insights into the origin and spread of this genotype. Studies of 142 European isolates revealed that those with the TR$_{34}$/L98H resistance mechanism showed less genetic variation than azole-susceptible isolates or those with a different genetic basis of resistance and were assigned to only four CSP (putative cell surface protein) types. Sexual crossing experiments demonstrated that TR$_{34}$/L98H isolates could outcross with azole-susceptible isolates of different genetic backgrounds, suggesting that TR$_{34}$/L98H isolates can undergo the sexual cycle in nature. Overall, our findings suggest a common ancestor of the TR$_{34}$/L98H mechanism and subsequent migration of isolates harboring TR$_{34}$/L98H across Europe.
**Introduction**

*Aspergillus fumigatus* is a saprophytic fungus that is capable of causing a wide range of diseases in various hosts. Invasive aspergillosis is the most severe manifestation of *Aspergillus* infection in humans, and this disease is associated with substantial mortality and morbidity. Medical triazoles, such as itraconazole, voriconazole, and posaconazole, play an important role in the management of *Aspergillus* diseases. However, azole resistance is an emerging problem in *A. fumigatus* and has been shown to be associated with increased probability of treatment failure [1-9].

Azole resistance is commonly due to mutations in the *cyp51A* gene, which encodes 14-α-demethylase in the ergosterol biosynthesis pathway. In azole-resistant clinical *A. fumigatus* isolates, a wide variety of *cyp51A* mutations, such as substitutions at codons G54, G138, P216, F219, M220, and G448, have been found [2,10-11]. This is in contrast with a different pattern of resistance observed in isolates from The Netherlands. Here, a resistance mechanism consisting of the L98H substitution together with a 34-bp tandem repeat (TR$_{34}$) in the promoter region of this gene (TR$_{34}$/L98H) was found to be present in over 90% of itraconazole-resistant isolates, which also showed reduced susceptibility to voriconazole and posaconazole [5,12]. TR$_{34}$/L98H isolates were recovered primarily from azole-naïve patients and were also recovered from the environment [12-13]. These observations suggest that azole-resistant *Aspergillus* is acquired by patients from an environmental source rather than arising through azole therapy. Recently, we provided evidence that exposure of *A. fumigatus* to 14-α-demethylase inhibitor (DMI) fungicides might provide a selective pressure leading to the emergence of TR$_{34}$/L98H resistant isolates in the environment [14]. On the basis of *in vitro* cross-resistance, molecule alignment studies, and docking simulations, five triazole fungicides that were highly similar to antifungal triazoles used in medicine were identified [14]. The TR$_{34}$/L98H resistance mechanism has been shown to be endemic in The Netherlands [12] and is also increasingly being reported in other European countries [2,5,15-17]. Furthermore, the TR$_{34}$/L98H genotype was recently reported outside Europe, in China and India [18-19].

At present, it is unknown if the high frequency of the TR$_{34}$/L98H genotype in azole-resistant isolates is due to migration from a common ancestral lineage or repeated independent development in genetically unrelated strains. Preliminary genotyping studies in TR$_{34}$/L98H isolates using microsatellites showed shorter genetic distances between TR$_{34}$/L98H isolates than those between wild-type controls [5], and another study showed that TR$_{34}$/L98H isolates nest within a single population and have not spread across *A. fumigatus* populations [20]. To gain more insights into the origin and spread of the TR$_{34}$/L98H resistance mechanism, we studied the genetic relatedness of European *A. fumigatus* isolates containing the TR$_{34}$/L98H mutations by analyzing several genetic markers. We also assessed the possible involvement of the recently described sexual cycle of *A. fumigatus* [21] in generating novel genetic diversity among isolates bearing the TR$_{34}$/L98H genotype.
Materials and methods

Selection of TR$_{34}$/L98H and control isolates

*A. fumigatus* isolates containing the TR$_{34}$/L98H resistance mechanism and originating between 1998 (the year in which the first TR$_{34}$/L98H isolates were recovered in The Netherlands) [5] and 2007 were selected from the fungal culture collection of the Radboud University Medical Centre. Dutch TR$_{34}$/L98H isolates, including isolates of clinical and environmental origin, were randomly selected. As controls, for each TR$_{34}$/L98H isolate, an isolate with a susceptible phenotype, which was matched by year of isolation to the TR$_{34}$/L98H isolate, was randomly selected. The controls also included both clinical and environmental isolates. To compare the TR$_{34}$/L98H resistance mechanism with other resistance mechanisms, the culture collection was also searched for isolates with an azole-resistant phenotype that was not due to the TR$_{34}$/L98H mechanism. Finally, TR$_{34}$/L98H A. *fumigatus* isolates that originated from other European countries were included (Table S1, available at http://jcm.asm.org/content/50/8/2674/suppl/DCSupplemental).

*In vitro* susceptibility testing

*In vitro* activity of itraconazole, voriconazole, and posaconazole was tested according to the EUCAST broth microdilution reference method [22]. The MIC was determined by the lowest antifungal concentration with a complete inhibition of growth after 48 h. MICs were interpreted based on recently proposed breakpoints [23].

DNA extraction and cyp51A sequencing

DNA was isolated, and the full coding sequence of the cyp51A gene, as well as the promoter region, was determined by amplification and subsequent sequencing as previously described [10]. Sequences were compared to a wild-type *A. fumigatus* cyp51A gene sequence (GenBank, National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/], accession number AF338659) to detect mutations.

CSP typing, microsatellite analysis, and mating type determination

CSP (putative cell surface protein) types were determined by PCR amplification and subsequent sequencing by following established protocols [24-25]. CSP types were assigned according to the CSP typing nomenclature described by Klaassen et al. [26].

Six microsatellite loci (STRAf 3A, 3B, 3C, 4A, 4B, and 4C) were analyzed as described previously [13,27]. The obtained genotypes were imported into BioNumerics v6.0 (Applied Maths, Sint-Martens-Latem, Belgium). A minimum spanning tree (MST) was constructed based on categorical treatment of the data, i.e., alleles were scored as either identical or nonidentical.
and the difference in numbers of repetitions at each locus between different genotypes was not taken into account.

Mating types were determined by a multiplex PCR-based mating type test, as described previously [28]. The null hypothesis of a 1:1 ratio of the two mating types was tested using the $\chi^2$ test, with a $P$ value of <0.05 considered significant [21].

**Sexual reproduction**

To investigate the segregation of genetic markers and the TR$_{34}$/L98H resistance mechanism following sexual reproduction, the wild-type isolate AfIR974 [21] was crossed with the clinical isolate v23-66, which harbored the TR$_{34}$/L98H resistance mechanism. Mating experiments were performed on oatmeal agar plates (Difco oatmeal agar), and inoculations were performed as described previously [21]. Plates were sealed with Parafilm and incubated at 30°C in the dark. Crosses were examined weekly, and when cleistothecia developed, ascospore suspensions were obtained as described previously [21]. Ascospore suspensions were plated onto Sabouraud agar plates, and germinating ascospores were transferred to Sabouraud agar slants. Fifteen progeny were selected for further analysis, including *in vitro* susceptibility testing and mating type determination. In addition, the progeny were screened for the presence of the TR$_{34}$/L98H resistance mechanism using the following PCR-based assays. The presence of the TR was investigated by amplifying part of the promoter region of the *cyp51A* gene using appropriate primers (5′-TGAGTTAGGGGTATGGTATGCTGGA-3′ and 5′-AGCAAGGGAGAAGAAAGCAGT-3′). For the L98H substitution, two PCRs were performed: an L98-specific PCR (primers 5′-CCTCTTCCGACATTGCTGGA-3′ and 5′-TGACGGCAATCTTGCAATGTGTTTA-3′) and an L98H-specific PCR (primers 5′-ACGAGTTTATTCTCAACGGCAGAAGGACTC-3′ and 5′-TTCCGGTGAAATCGCGCAGATGCT-3′). The cycling program consisted of a 2-min denaturation step at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 60°C, and 45 s at 72°C and a final elongation step of 5 min at 72°C. Products of the TR detection PCR were separated on a 2% agarose gel together with a size marker. Amplicons of 188 bp in size do not contain a TR, while the presence of a TR will lead to amplicons of 222 bp (188 bp plus an extra 34-bp repeat). Amplification products of the L98- and L98H-specific PCRs were mixed and run on a 2% agarose gel with a size marker. The L98-specific product is 341 bp in size, while the L98H-specific product is 223 bp in size. After the first sexual cross with a TR$_{34}$/L98H isolate succeeded, additional TR$_{34}$/L98H isolates were subjected to sexual crossing. For that, AfIR957 (MAT1-1) [21] and AfIR928 (MAT1-2) [21,29] were crossed with various clinical isolates of the opposite mating type that contained the TR$_{34}$/L98H resistance mechanism. The progeny of each cross were assessed both for the presence of the TR$_{34}$/L98H mutations (by the PCR-based assays as described above) and for their CSP type.
Results

TR$_{34}$/L98H and control isolates
The distribution of isolates over time is shown in Table 1. In total, 55 azole-resistant TR$_{34}$/L98H isolates and 55 azole-susceptible wild-type controls that had been cultured between 1998 and 2007 in The Netherlands were selected. Of these, 86 were clinical isolates which originated from patients admitted to the Radboud University Nijmegen Medical Centre (71 isolates) or other Dutch hospitals (15 isolates). The remaining 24 isolates were of environmental origin and were recovered from soil, seeds, compost, leaves, water filter samples, and air samples (see Table S1, available at http://jcm.asm.org/content/50/8/2674/suppl/DCSupplemental).

The fungal culture collection comprising over 2,000 isolates of A. fumigatus contained seven resistant isolates without the TR$_{34}$/L98H resistance mechanism that were cultured between 1998 and 2007 in The Netherlands (Table 1). Three isolates were of clinical origin and harbored a point mutation in the cyp51A gene, leading to the M220K, M220I, or M220V substitution. These mutations are known to be correlated with azole resistance [30-32]. The other four azole-resistant isolates were obtained from the environment and did not have any mutation in the cyp51A gene.

Table 1. Distribution of azole-resistant and azole-susceptible wild-type Dutch A. fumigatus isolates examined in this study according to the year of isolation.

<table>
<thead>
<tr>
<th>Year of isolation</th>
<th>No. of isolates with each phenotype and resistance mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
</tr>
<tr>
<td>1998</td>
<td>2</td>
</tr>
<tr>
<td>2000</td>
<td>2</td>
</tr>
<tr>
<td>2001</td>
<td>2</td>
</tr>
<tr>
<td>2002</td>
<td>7</td>
</tr>
<tr>
<td>2003</td>
<td>4</td>
</tr>
<tr>
<td>2004</td>
<td>7</td>
</tr>
<tr>
<td>2005</td>
<td>6</td>
</tr>
<tr>
<td>2006</td>
<td>7</td>
</tr>
<tr>
<td>2007</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
</tr>
</tbody>
</table>
**In vitro susceptibility testing**

Results of in vitro susceptibility testing of the Dutch isolates are shown in Table 2. According to the proposed breakpoints [23], all of the 55 TR$_{34}$/L98H isolates were resistant to itraconazole and all except two showed resistance or intermediate susceptibility to voriconazole. Ten isolates were susceptible to posaconazole, while the other 45 showed intermediate susceptibility or resistance to this drug. Of the seven non-TR$_{34}$/L98H resistant isolates exhibiting resistance to azoles, five were itraconazole resistant, and of these, four isolates also showed intermediate or resistant phenotypes for voriconazole and/or posaconazole. The remaining two isolates were resistant to voriconazole, with a MIC of 4 mg/liter, while being susceptible to both itraconazole and posaconazole.

<table>
<thead>
<tr>
<th>Resistance mechanism (number of isolates)</th>
<th>MIC geometric mean (range) (mg/liter)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITZ</td>
</tr>
<tr>
<td>Wild-type (55)</td>
<td>0.19 (0.031-1)</td>
</tr>
<tr>
<td>TR$_{34}$/L98H (55)</td>
<td>26.82 (4-32)</td>
</tr>
<tr>
<td>non-TR$_{34}$/L98H (7)</td>
<td>8.83 (0.5-32)</td>
</tr>
</tbody>
</table>

$^a$For the purpose of the analysis, all values >16 mg/l were indicated as 32 mg/l.

ITZ, itraconazole; VOR, voriconazole; POS, posaconazole.

**CSP genotyping and mating type**

CSP typing showed that the azole-susceptible wild-type control isolates were spread across 11 CSP types (Table 3). The seven Dutch azole-resistant non-TR$_{34}$/L98H isolates were distributed over CSP types t01, t02, t03, and t04A. In contrast, the 55 Dutch isolates with the TR$_{34}$/L98H resistance mechanism grouped in only three CSP types: t02, t04B, and t11. Remarkably, CSP types t04B and t11 consisted exclusively of TR$_{34}$/L98H isolates, while t02 types contained azole-susceptible and TR$_{34}$/L98H and non-TR$_{34}$/L98H resistant isolates. CSP types t02, t04B, and t11 all contained clinical as well as environmental TR$_{34}$/L98H isolates. The distribution of CSP types over time within the group of TR$_{34}$/L98H isolates is shown in Figure 1. The first Dutch TR$_{34}$/L98H isolates that were recovered from a patient in 1998 were of CSP type t11. TR$_{34}$/L98H isolates of a second and third CSP type were then found in 2000 and 2002 (t04B and t02, respectively). From 2002 until 2007, no further new CSP types were observed among the Dutch TR$_{34}$/L98H isolates.

The multiplex PCR showed isolates of complementary MAT1-1 and MAT1-2 mating types to be present in all isolate groups; within the 55 wild-type isolates, a distribution ratio of 36%
to 64% was found for MAT1-1/MAT1-2, while in the 55 TR$_{34}$/L98H isolates, a ratio of 55% to 45% was detected, and in the final 7 non-TR$_{34}$/L98H resistant isolates, a ratio of 57% to 43% for MAT1-1/MAT1-2 was found. There was no significant deviation from a 1:1 ratio in any of these groups according to a $\chi^2$ statistical analysis. With regard to CSP types, TR$_{34}$/L98H isolates of both MAT1-1 and MAT1-2 mating types were present within all three CSP types (t02, t04B, and t11) (data not shown).

**Table 3.** Distribution of CSP types in the Dutch and European *A. fumigatus* isolates.

<table>
<thead>
<tr>
<th>CSP type</th>
<th>Dutch wild-type (55)</th>
<th>Dutch resistant non-TR$_{34}$/L98H (7)</th>
<th>Dutch resistant TR$_{34}$/L98H (55)</th>
<th>Other European TR$_{34}$/L98H (25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t01</td>
<td>15 (27.3)</td>
<td>4 (57.1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t02</td>
<td>4 (7.3)</td>
<td>1 (14.3)</td>
<td>12 (21.8)</td>
<td>12 (48)</td>
</tr>
<tr>
<td>t03</td>
<td>7 (12.7)</td>
<td>1 (14.3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t04A</td>
<td>16 (29.1)</td>
<td>1 (14.3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t04B</td>
<td>-</td>
<td>-</td>
<td>20 (36.4)</td>
<td>8 (32)</td>
</tr>
<tr>
<td>t05</td>
<td>2 (3.6)</td>
<td>-</td>
<td>-</td>
<td>1 (4)</td>
</tr>
<tr>
<td>t08</td>
<td>5 (9.1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t09</td>
<td>1 (1.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t10</td>
<td>1 (1.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t11</td>
<td>-</td>
<td>-</td>
<td>23 (41.8)</td>
<td>4(16)</td>
</tr>
<tr>
<td>t13</td>
<td>2 (3.6)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t14</td>
<td>1 (1.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>t18</td>
<td>1 (1.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Molecular epidemiology of isolates harboring the TR$_{34}$/L98H mechanism

Figure 1. Distribution of CSP types of 55 TR$_{34}$/L98H A. fumigatus isolates isolated between 1998 and 2007 in The Netherlands.

Other European TR$_{34}$/L98H isolates
The Radboud fungus culture collection contained 25 TR$_{34}$/L98H isolates from seven other European countries (Austria, Belgium, Denmark, France, Italy, Norway, and United Kingdom) (see Table S1, available at http://jcm.asm.org/content/50/8/2674/suppl/DCSupplemental). Only the isolate from Norway was of environmental origin; the other 24 isolates were of clinical origin. CSP typing of these TR$_{34}$/L98H isolates showed that they grouped to the same CSP types as the TR$_{34}$/L98H isolates originating from The Netherlands (i.e., t02, t04B, and t11). There was only one exception: one of the five TR$_{34}$/L98H isolates originating from Italy was of CSP type t05 (Table 3).

Microsatellite typing of Dutch and European isolates
A minimum spanning tree (MST) was constructed based on the six STRAf microsatellite loci, with the majority of isolates having a unique genotype (Figure 2). The Dutch TR$_{34}$/L98H isolates formed a cluster that was almost entirely separate from the azole-susceptible wild-type isolates. In three cases, a clinical and an environmental TR$_{34}$/L98H isolate had identical microsatellite genotypes.

The TR$_{34}$/L98H isolates from other European countries also clustered with the Dutch TR$_{34}$/L98H isolates. TR$_{34}$/L98H isolates of two particular microsatellite genotypes were found in both The Netherlands and other European countries (see the dual-colored dots in Figure 2). In addition, isolates from Belgium and Italy also shared the same genotype. The non-TR$_{34}$/L98H resistant isolates did not cluster together and instead were more distributed throughout the tree.
Figure 2. Minimum spanning tree showing the genotypic relationship between the azole-resistant and azole-susceptible *A. fumigatus* isolates.

Each circle corresponds to a unique genotype, and the size of the circle corresponds to the number of isolates with that genotype (1, 2 or 3 isolates). Connecting lines correspond to the number of different microsatellite loci between the genotypes. Short bold line, 1 difference; black line, 2 differences; long grey line, 3 differences; dotted line, 4 or more differences.

Red, azole-resistant TR<sub>R</sub>/L98H, The Netherlands (n = 55); yellow, azole-susceptible wild-type, The Netherlands (n = 55); blue, azole-resistant non-TR<sub>R</sub>/L98H, The Netherlands (n = 7); pink: azole-resistant TR<sub>R</sub>/L98H, other European countries (n = 25).
Sexual crosses with TR_{34}/L98H isolates

In order to gain insight into the impact of sexual reproduction on the genetic markers, the wild-type isolate AfIR974 (MAT1-1, CSP type t02) was crossed with the clinical isolate v23-66, containing the TR_{34}/L98H resistance mechanism (MAT1-2, CSP type t04B). After approximately 2 months of incubation, cleistothecia were formed and ascospores were isolated. As shown in Table 4, 15 progeny, of which eight (53%) harbored the TR_{34}/L98H resistance mechanism and seven did not, were analyzed. The presence of TR_{34}/L98H always corresponded with an azole-resistant phenotype. The presence of new combinations of both mating and CSP types in the progeny and the generation of new microsatellite genotypes provided evidence for recombination during the heterothallic sexual cycle [21]. The combination of all genotypic markers (presence of TR_{34}/L98H, six STRAf microsatellites, CSP and mating type) resulted in unique genotypes for 80% of the progeny. Surprisingly, in two of the progeny (AfIR974-v23-66-10 and AfIR974-v23-66-14), the number of repeats in the STRAf 3A marker increased from 80 to 82, a result which is suggestive of microvariation [33-34].

In addition, six TR_{34}/L98H isolates were subjected to sexual crossing to determine whether they could outcross with isolates from a different CSP type that was not observed among the Dutch TR_{34}/L98H resistant isolates. Clinical TR_{34}/L98H isolates of CSP types t02, t04B, and t11 and of each mating type were crossed to the environmental isolates AfIR957 (MAT1-1, CSP type t05) and AfIR928 (MAT1-2, CSP type t03). Four out of six crosses were fertile (see Table S2, available at http://jcm.asm.org/content/50/8/2674/suppl/DCSupplemental). Analysis of the progeny from each successful cross revealed the presence of isolates that were of the TR_{34}/L98H genotype and of CSP types t03 and t05 (see Table S2). This demonstrated that the presence of the TR_{34}/L98H mechanism is not necessarily restricted to CSP types t02, t04B, and t11.
**Table 4.** Genotypes and *in vitro* azole susceptibility profiles of the parental isolates and 15 ascospore progeny from a cross between an azole-resistant TR₃₄/L98H *A. fumigatus* isolate (v23-66) and an azole-susceptible wild-type isolate (AfIR974).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (mg/liter)</th>
<th>TR₃₄ presence</th>
<th>Codon 98</th>
<th>Mating type</th>
<th>CSP type</th>
<th>No. of repeats of each STRAf microsatellite</th>
<th>Geno-type&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AfIR974</td>
<td>0.25</td>
<td>0.5</td>
<td>0.063</td>
<td>-</td>
<td>L98</td>
<td>34 9 10 8 10 30</td>
<td>A</td>
</tr>
<tr>
<td>v23-66</td>
<td>&gt;16</td>
<td>4</td>
<td>0.5</td>
<td>+</td>
<td>L98H</td>
<td>80 9 9 8 10 11</td>
<td>B</td>
</tr>
<tr>
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<td>&gt;16</td>
<td>4</td>
<td>0.5</td>
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<td>L98H</td>
<td>34 9 10 8 10 11</td>
<td>C</td>
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<tr>
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<td>0.5</td>
<td>0.063</td>
<td>-</td>
<td>L98</td>
<td>80 9 10 8 10 30</td>
<td>D</td>
</tr>
<tr>
<td>AfIR974-v23-66-3</td>
<td>&gt;16</td>
<td>4</td>
<td>0.5</td>
<td>+</td>
<td>L98H</td>
<td>34 9 10 8 10 11</td>
<td>E</td>
</tr>
<tr>
<td>AfIR974-v23-66-4</td>
<td>&gt;16</td>
<td>2</td>
<td>0.5</td>
<td>+</td>
<td>L98H</td>
<td>80 9 10 8 10 11</td>
<td>F</td>
</tr>
<tr>
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<td>&gt;16</td>
<td>4</td>
<td>0.5</td>
<td>+</td>
<td>L98H</td>
<td>34 9 10 8 10 30</td>
<td>C</td>
</tr>
<tr>
<td>AfIR974-v23-66-6</td>
<td>&gt;16</td>
<td>4</td>
<td>0.5</td>
<td>+</td>
<td>L98H</td>
<td>80 9 9 8 10 30</td>
<td>G</td>
</tr>
<tr>
<td>AfIR974-v23-66-7</td>
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<td>0.5</td>
<td>0.063</td>
<td>-</td>
<td>L98</td>
<td>80 9 9 8 10 30</td>
<td>H</td>
</tr>
<tr>
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<td>0.5</td>
<td>0.063</td>
<td>-</td>
<td>L98</td>
<td>80 9 9 8 10 30</td>
<td>I</td>
</tr>
<tr>
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<td>0.5</td>
<td>0.063</td>
<td>-</td>
<td>L98</td>
<td>80 9 9 8 10 30</td>
<td>J</td>
</tr>
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<td>4</td>
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<td>80 9 9 8 10 30</td>
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<td>-</td>
<td>L98</td>
<td>80 9 9 8 10 30</td>
<td>L</td>
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<tr>
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<td>4</td>
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<td>80 9 9 8 10 30</td>
<td>M</td>
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<tr>
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<td>0.5</td>
<td>+</td>
<td>L98H</td>
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<td>N</td>
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<tr>
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<td>0.063</td>
<td>-</td>
<td>L98</td>
<td>80 9 9 8 10 30</td>
<td>A</td>
</tr>
</tbody>
</table>

<sup>a</sup>ITZ, itraconazole; VOR, voriconazole; POS, posaconazole.

<sup>b</sup>The different genotypes of the progeny isolates, defined by unique combinations of the presence/absence of TR₃₄/L98H, mating type, CSP type, and six microsatellite markers, are identified by different letters of the alphabet.
Discussion

There are an increasing number of studies reporting the TR\textsubscript{34}/L98H mutations as a key underlying resistance mechanism in azole-resistant \textit{A. fumigatus} isolates. The TR\textsubscript{34}/L98H mechanism was found to be widespread in The Netherlands and is also found in other European and Asian countries [2,5,15-16,18-19,35-37]. TR\textsubscript{34}/L98H isolates have been recovered from the environment in The Netherlands, Denmark, and Norway [5,13,16], and there is increasing evidence that this resistance mechanism has developed through a fungicide-driven route of resistance development [14]. The present study was undertaken to gain insights into the origin and spread of this resistance genotype.

The first key finding was that isolates with the TR\textsubscript{34}/L98H azole resistance mechanism originating from The Netherlands and seven other European countries are genetically less diverse than azole-susceptible wild-type isolates and isolates bearing other forms of azole resistance (for the latter two groups, only isolates originating from The Netherlands were tested). The two genotyping methods used, CSP and microsatellites, gave different levels of discrimination. CSP typing has a lower discriminatory power than microsatellite analysis and has been suggested to be more suitable for typing at the subpopulation level [26]. This typing method revealed the significant result that Dutch isolates with the TR\textsubscript{34}/L98H genotype were found only in three CSP types, namely, t02, t04B, and t11. This contrasted with the distribution of CSP types in the azole-susceptible wild-type control group from The Netherlands, where isolates were found in 11 different CSP groups, similar to previously published results [25-26]. Importantly, two CSP types (t04B and t11) were observed to comprise exclusively TR\textsubscript{34}/L98H isolates, with no non-TR\textsubscript{34}/L98H resistant or azole-susceptible isolates found within these groups. This is consistent with a previous report in which isolates from Australia (where TR\textsubscript{34}/L98H-mediated resistance has not been described to date) were analyzed which also failed to detect isolates of these two CSP types [25]. However, the absence of azole-susceptible isolates in these CSP types may simply be due to a very low frequency of occurrence. Meanwhile, 24 out of 25 TR\textsubscript{34}/L98H isolates (96%) of European origin other than The Netherlands grouped to the same three CSP types, indicating a close genetic relationship despite the geographic distances involved. Results of the microsatellite analysis were consistent with the CSP typing, demonstrating a clustering of all TR\textsubscript{34}/L98H isolates, distinct from most wild-type, azole-susceptible isolates. This was also shown in collections of Dutch isolates [5,13]. Another study concluded that resistant clinical isolates were more distributed among susceptible isolates, although most TR\textsubscript{34}/L98H isolates clustered within the same clade of the phylogenetic tree [38]. From the data presented here, a close genetic relationship between all European TR\textsubscript{34}/L98H isolates can be inferred. However, it should be cautioned that as no azole-susceptible isolates from European countries other than The Netherlands were included in the analysis,
we can conclude only that the TR$_{34}$/L98H grouping is distinct specifically from the Dutch azole-susceptible isolates.

Considering the CSP typing and microsatellite results as a whole, it is possible to speculate about the evolutionary origin and spread of the TR$_{34}$/L98H azole resistance genotype within Europe. The relatively close genetic relationships and limited CSP type diversity of the TR$_{34}$/L98H isolates, compared to those of the azole-susceptible wild-type isolates, indicates that the independent and repeated emergence of the TR$_{34}$/L98H mechanism seems unlikely. An alternative explanation is that the TR$_{34}$/L98H isolates developed from a common ancestor or restricted set of genetically related isolates. A common origin of the TR$_{34}$/L98H mechanism was previously suggested based on the microsatellite typing of 144 Dutch TR$_{34}$/L98H isolates, from which it was calculated that the first TR$_{34}$/L98H isolate would have emerged in the Netherlands in 1997 [14]. The appearance and spread of the TR$_{34}$/L98H mutation from an apparent common source is in marked contrast to the evolution of the non-TR$_{34}$/L98H resistant isolates detected in the present study. These were distributed throughout the total set of isolates (Figure 2), consistent with the hypothesis that mutations, such as at codon M220 in cyp51A, can be induced in isolates through prolonged patient exposure to medical triazoles [39]. One TR$_{34}$/L98H isolate from Italy was of CSP type t05, but it still grouped with the other TR$_{34}$/L98H isolates in the microsatellite analysis. Further work is required to determine if CSP type t05 azole-resistant isolates are widespread in Italy and/or have spread to other countries.

A second key finding of the present study was that sexual reproduction, recently discovered in A. fumigatus [21], can occur between isolates of different CSP types and TR$_{34}$/L98H genotypes to give rise to isolates with novel combinations of the TR$_{34}$/L98H resistance mechanism in other CSP types. The finding that TR$_{34}$/L98H isolates are sexually fertile provides a possible explanation for their genetic diversity. It is hypothesized that very few sexual cycles in the field would give rise to progeny exhibiting the degree of CSP variation observed in this study. Indeed, this is supported by the presence of both MAT1-1 and MAT1-2 isolates in the t02, t04B, and t11 CSP types. The accessory role of sexual reproduction was also suggested by a recent report which found that the TR$_{34}$/L98H genotype was nested within a single, predominantly asexual population [20]. Figure 1 suggests that sexual reproduction played a role in the early stages of development of azole resistance, as all three CSP types were already present in TR$_{34}$/L98H isolates in The Netherlands by the year 2002. Moreover, the Norwegian isolate from 2000, not included in the analysis of Dutch isolates, grouped to CSP type t02, indicating that all three CSP types were already present in the initial years of TR$_{34}$/L98H emergence. After sexual reproduction, subsequent asexual reproduction would most probably lead to persistence of these isolates and CSP types, given that after 100 (in vitro) asexual generations, no variations in CSP type were found (C.H.W. Klaassen, unpublished data). In the meantime, the number of repeats in microsatellite markers might undergo
subtle changes during asexual reproduction (C.H.W. Klaassen, unpublished data), leading to the observed diversity in microsatellite markers.

Overall, our findings suggest a common origin of the TR34/L98H mechanism and the subsequent migration of TR34/L98H isolates across Europe. A similar spread of fungicide resistance has been shown before in plant pathogens: in the wheat pathogen *Mycosphaerella graminicola*, mutations causing resistance arose locally and were then spread across Europe through wind-dispersed ascospores [37,40], and in the grape pathogen *Botrytis cinerea*, resistant isolates probably migrated from French to German wine-growing regions [41]. More research into the genesis of the TR34/L98H mechanism is needed in order to understand the conditions that facilitate azole resistance development in the environment so that measures can be implemented to prevent the emergence of new resistance mechanisms.

Acknowledgements

We thank all international and national collaborators for sending *A. fumigatus* isolates.
Chapter 5

References


Molecular epidemiology of isolates harboring the TR$_{34}$/L98H mechanism


Molecular epidemiology of isolates harboring the TR_{34}/L98H mechanism


Microscopic image of a Blankophor stained postmortem kidney swab sample, obtained from a renal transplant patient with invasive aspergillosis (picture: Diane Lamers-Jansen).
A new *cyp51A*-mediated resistance mechanism that confers voriconazole resistance in clinical and environmental *Aspergillus fumigatus* isolates

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*contributed equally to this publication
Abstract

Azole-resistance is an emerging problem in *Aspergillus fumigatus* and complicates the management of patients with *Aspergillus* 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*. Clinical *A. fumigatus* isolates were screened for azole resistance in eight University Hospitals using azole agar dilution plates. Patient information was collected and azole-resistant *A. fumigatus* isolates were analyzed using gene sequencing, susceptibility testing and genotyping. Recombination experiments were performed to link identified mutations to resistance phenotypes. Air sampling was performed to investigate the presence of resistant isolates in the environment.

Between December 2009 and January 2011 1,315 *A. fumigatus* isolates from 921 patients were screened. A new Cyp51A-mediated resistance mechanism (TR<sub>46</sub>/Y121F/T289A) emerged in 21 azole-resistant isolates from 15 patients in six hospitals. Eleven patients had no history of previous azole exposure. TR<sub>46</sub>/Y121F/T289A isolates were highly resistant to voriconazole (MIC ≥16 mg/l). Eight patients presented with invasive aspergillosis, and all five patients receiving primary therapy with voriconazole failed. Recombinant experiments confirmed the link between TR<sub>46</sub>/Y121F/T289A and the resistant phenotype. TR<sub>46</sub>/Y121F/T289A was recovered from 6 of 10 sampled environmental sites.

We describe the emergence and migration of a new pan-azole resistance mechanism in *A. fumigatus*. The similar epidemiology and clinical characteristics of TR<sub>46</sub>/Y121F/T289A to the TR<sub>34</sub>/L98H resistance mechanism point towards a fungicide-driven route of resistance selection. We believe that the current use of azole fungicides in our environment has serious side effects, such as the continued selection and migration of resistance traits in *A. fumigatus* and the loss of the azole class for the management of patients with *Aspergillus* 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 mould *Aspergillus fumigatus*. These triazoles are clinically licensed for the prevention and treatment of both non-invasive *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 where 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 and material preservation. Five DMIs showed *in vitro* activity against *A. fumigatus*, and were, similar to the clinically licensed azoles, from the triazole class [11]. The environmental mode of resistance development is of major importance as over 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\(_{34}\)/L98H). DMIs that exhibit similar docking poses to the medical triazoles showed complete loss of activity against *A. fumigatus* isolates that harbor TR\(_{34}\)/L98H, giving further evidence for a possible environmental route of resistance development in *A. fumigatus* [11].

TR\(_{34}\)/L98H first emerged in Dutch clinical *A. fumigatus* isolates in 1998 and a National surveillance study indicated that this resistance mechanism is now endemic in Dutch hospitals and infections may occur in azole-treated as well as in azole-naïve patients [6,7]. TR\(_{34}\)/L98H is increasingly reported in other European countries, and more recently also in China and India [6,13-21]. Molecular typing studies indicate that the fungicide-driven route of resistance development carries the risk of migration of this resistance trait, similar to azole-resistant phytopathogenic fungi [22].

In our current study we investigated the emergence of a new azole resistance mechanism in *A. fumigatus*. Our hypothesis was that this new azole resistance mechanism has emerged through an environmental route of resistance selection. We investigated the underlying resistance mechanism, describe the epidemiology and clinical implications, and performed environmental sampling to determine if the new resistance mechanism was present in our environment.
Chapter 6

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 eight University Medical Centres in the Netherlands were routinely screened for the presence of azole-resistance, irrespective of the clinical relevance of the culture result. An on-line questionnaire was completed in seven out of eight centres for every collected isolate, which included questions about isolate and patient characteristics. In one centre the questionnaire was only fulfilled for resistant isolates.

Screening: four-well azole-agar dilution plates
Aspergillus colonies that grew in primary cultures were subcultured on a specially developed four-well azole-agar dilution plate (4D-plate) [23]. All wells contained RPMI-1640 agar and three wells were each supplemented with one 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, i.e. the ability to grow at 48°C and macro- and microscopic culture morphology.

Phenotypic analyses
All A. fumigatus isolates that grew on one or more azole-containing agar wells were investigated for their antifungal susceptibility to itraconazole, voriconazole, posaconazole, and the DMI tebuconazole using the CLSI M38-A2 broth microdilution reference method [24].

Genotypic analyses
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 PCR amplification and sequencing to detect any mutations (reference cyp51A sequence: GenBank AF338659) [25]. Molecular identification was performed by sequencing the highly conserved β-tubulin and calmodulin gene, as described previously [26]. Microsatellite genotyping was used to investigate genetic distances between the isolates by
Voriconazole resistance from clinical and environmental origin

analysis of six microsatellites (STRAf 3A, 3B, 3C, 4A, 4B, and 4C), as described previously [6]. If multiple resistant isolates were obtained from one patient, only the first isolate was included. For every resistant isolate, two control isolates were selected that had been cultured between one month before and one month after the date of isolation of the resistant isolate. One control isolate harbored the TR$_{34}$/L98H resistance mechanism, while 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 to produce the dendrogram [27].

Cyp51A homology model
The locations of the observed amino acid substitutions of the presumed novel resistance mechanism were investigated with help of a structural model of the $A. fumigatus$ Cyp51A enzyme. This homology model was described previously and was constructed based on the human Cyp51 crystal structure [11].

A. fumigatus recombinants
To confirm the link of the presumed new resistance mechanism with the azole-resistant phenotype, $A. fumigatus$ recombinants were created as described before [28]. Alterations were introduced into a cyp51A cassette and subsequently the mutated cassette was electroporated into a susceptible $A. fumigatus$ transformation recipient isolate. Susceptibility testing of the recombinants, the transformation recipient and transformation control isolate (i.e. $A. fumigatus$ transformed with a wildtype cassette) was performed as described above.

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 for those patients with cancer [29]. 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 domestic homes (six in the Nijmegen area and
two in the Groningen area). Indoor sites and one site in the direct outdoor proximity were sampled. Air samples were obtained using an air sampler type Casella (Cat No E7627/Z-24). 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/min; 20 min) to detect azole-resistant spores. The plates were incubated at 37°C and inspected twice daily for four 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. *A. 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 [22]. Tandem repeats of different sizes could be identified on the basis of the size of the amplified PCR fragment.

Isolates containing the TR\(^{34}\) were screened for the presence of the L98H substitution by using two PCR reactions: a L98- and a L98H specific PCR (primers described elsewhere) [22]. Azole-resistant isolates without TR\(^{34}/L98H\) were selected for sequencing the *cyp51A* gene- and promoter region as described above [25].

**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 (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 two mutations leading to substitutions Y121F and T289A. In addition, a 46 base pair tandem repeat was found in the gene promoter (TR\(^{46}/Y121F/T289A\)). A second isolate was cultured in January 2010 from a patient in Amsterdam which exhibited a similar voriconazole-resistant phenotype and identical TR\(^{46}/Y121F/T289A\) 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, five 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 TR\(^{46}/Y121F/T289A\) resistance mechanism, that had been cultured on December 31\(^{st}\) 2009 in Utrecht. This was considered to be the first clinical isolate from our surveillance network to harbor the TR\(^{46}/Y121F/T289A\) resistance mechanism.
Prevalence of the TR$_{46}$/Y121F/T289A resistance mechanism

From December 2009 to January 2011, 1,315 A. fumigatus isolates from 921 patients were screened for resistance in seven out of eight university centres. In one centre the total number of isolates screened was unknown, as they did not complete the on-line questionnaire for their susceptible isolates. The overall prevalence of azole resistance was 6.8% (63 of 921 patients). 47 of 63 patients (74.6%) harbored the TR$_{34}$/L98H resistance mechanism and 13 patients (20.6%) TR$_{46}$/Y121F/T289A. No cyp51A mutations were found in azole-resistant A. fumigatus isolates from three patients (4.7%). The prevalence of TR$_{46}$/Y121F/T289A was 1.4% (13 of 921 patients) in this 14 month period. Besides this, two isolates with TR$_{46}$/Y121F/T289A and two isolates with TR$_{34}$/L98H were recovered from the centre which had not recorded the total number of screened isolates. Therefore, within 14 months (December 2009 to January 2011) TR$_{46}$/Y121F/T289A was detected in 21 clinical A. fumigatus isolates obtained from 15 patients in six different University hospitals in the Netherlands (i.e. Amsterdam (2 hospitals), Groningen, Leiden, Nijmegen, Utrecht) (Table 1).

Characterization of TR$_{46}$/Y121F/T289A isolates

The 21 TR$_{46}$/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 the majority of the isolates (Table 1). The DMI tebuconazole, which has been shown to exhibit activity against wild-type A. fumigatus isolates, showed no in vitro activity against TR$_{46}$/Y121F/T289A isolates. One isolate with TR$_{46}$/Y121F/T289A contained two additional substitutions; M172I and G448S.

The location of Y121 and T289 in the Cyp51A protein structure was checked based on the Cyp51A homology model. The model showed that Y121 was in direct contact with the haem group in the centre of the enzyme, while T289 was situated in one of the two ligand access channels (Figure 1).

A. fumigatus recombinants were constructed with the presumed TR$_{46}$/Y121F/T289A resistance mechanism (Table 2). As shown in Table 3, the TR$_{46}$/Y121F/T289A recombinants showed no susceptibility of voriconazole, while the MIC of posaconazole increased from <0.031 to 0.25-0.5 mg/l. For itraconazole the MIC appeared to remain similar to that of the control isolate. Tebuconazole was active against the wild-type recipient isolate, but showed no activity against the TR$_{46}$/Y121F/T289A recombinant (Table 2).

Microsatellite genotyping showed that clinical TR$_{46}$/Y121F/T289A isolates clustered together. TR$_{46}$/Y121F/T289A and TR$_{34}$/L98H were separated into different clades and apart from wild type control isolates (Figure 2).
<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 [29]</th>
<th>Previous azole exposure*/**</th>
<th>Treatment*</th>
<th>Outcome at 12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>F / 11</td>
<td>Dec. 2009 / sputum</td>
<td>Utrecht</td>
<td>4  &gt;16  0.25</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>&gt;16 &gt;16  2</td>
<td>Chronic otitis externa, sinusitis and paralysis of abducens nerve</td>
<td>IA***</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>2 &gt;16 0.5</td>
<td>Kidney transplantation</td>
<td>Proven IA</td>
<td>None</td>
<td>VCZ, POS</td>
<td>Died</td>
</tr>
<tr>
<td>F / 9</td>
<td>Feb. 2010 / sputum</td>
<td>Amsterdam</td>
<td>4 &gt;16 0.5</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>&gt;16 &gt;16 2</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>1 &gt;16 0.25</td>
<td>Multiple myeloma, autol 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>16 &gt;16 0.5</td>
<td>Cystic fibrosis, bilateral lung transplantation</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>4 &gt;16 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>&gt;16 &gt;16 1</td>
<td>Lung fibrosis</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Alive</td>
</tr>
</tbody>
</table>

Table 1. Clinical characteristics of 15 patients from whom an *A. fumigatus* isolate was cultured that harbored the TR<sub>46</sub>/Y121F/T289A resistance mechanism.
### Voriconazole resistance from clinical and environmental origin

<table>
<thead>
<tr>
<th>Sex</th>
<th>Date</th>
<th>Location</th>
<th>Age</th>
<th>1/16</th>
<th>1/16</th>
<th>Resistance</th>
<th>Proven IA</th>
<th>Treatment</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Jun. 2010</td>
<td>Amsterdam</td>
<td>1</td>
<td>&gt;16</td>
<td>0.25</td>
<td>High energetic trauma, ICU admission</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>M</td>
<td>Jul. 2010</td>
<td>Amsterdam</td>
<td>4</td>
<td>&gt;16</td>
<td>1</td>
<td>B-thalassemia and diabetes mellitus</td>
<td>Proven IA</td>
<td>None</td>
<td>VCZ, L-AMB, CAS</td>
</tr>
<tr>
<td>F</td>
<td>Sep. 2010</td>
<td>Nijmegen</td>
<td>2</td>
<td>&gt;16</td>
<td>0.5</td>
<td>Cystic fibrosis</td>
<td>ABPA</td>
<td>VCZ</td>
<td>None</td>
</tr>
<tr>
<td>F</td>
<td>Oct. 2010</td>
<td>Groningen</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>2</td>
<td>COPD, unilateral lung transplantation</td>
<td>None</td>
<td>None</td>
<td>L-AMB, VCZ</td>
</tr>
<tr>
<td>F</td>
<td>Nov. 2010</td>
<td>Leiden</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>2</td>
<td>COPD</td>
<td>No IA</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>F</td>
<td>Jan. 2011</td>
<td>Utrecht</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>1</td>
<td>NH B-cell lymphoma, allo-SCT</td>
<td>Probable IA</td>
<td>VCZ</td>
<td>VCZ</td>
</tr>
</tbody>
</table>

*ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; L-AmB, lipid-formulation of amphotericin B; CAS, caspofungin; AND, anidulafungin.

**Azole exposure within 12 weeks preceding the culture of the azole resistant isolate.

***This patient could not be classified according to the EORTC/MSG consensus definitions. The patient showed bone destruction of the skull on CT scan and *A. fumigatus* was recovered repeatedly from the ear, without any other explanation.
Figure 1. The location of the Y121F and T289A substitutions in the Cyp51A homology model. The two ligand access channels are indicated in green.

Table 2. Primer sequences used to obtain the TR_{46}, Y121F and T289A alterations into the cyp51A cassette. TR_{46} was introduced by PCR amplification of the promoter region of a clinical isolate harboring this repeat and subsequently ligating it into the cassette, as was described before for TR_{34} [28]. Y121F and T289A were subsequently introduced by site-directed mutagenesis [28].

<table>
<thead>
<tr>
<th>Cyp51A alteration</th>
<th>Primer sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TR_{46}</td>
<td>GGAATAGACGCGGTTCACCA</td>
</tr>
<tr>
<td></td>
<td>GCCATACTGCCGGTTAAGAA</td>
</tr>
<tr>
<td>Y121F</td>
<td>TTCGGATCGGGACGTGGTTTGGATTGCATACATCGCATTCCAAATTC</td>
</tr>
<tr>
<td></td>
<td>GAATTGGGACCAATCAACACCGGTCGATGCCGAA</td>
</tr>
<tr>
<td>T289A</td>
<td>CCATCAACAGGGCTACATCATGTCGCAATCTCTTT</td>
</tr>
<tr>
<td></td>
<td>AAGAGATTGCGCAGATGATGACCGCCTTGTTGATGG</td>
</tr>
</tbody>
</table>
Table 3. Phenotype of the transformation recipient, transformation control without any mutations in the cassette and two TR<sub>46</sub>/Y121F/T289A recombinants obtained from two separate electroporation experiments.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (mg/l)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITZ</td>
</tr>
<tr>
<td>Transformation recipient</td>
<td>0.25</td>
</tr>
<tr>
<td>Transformation control</td>
<td>0.25</td>
</tr>
<tr>
<td>TR&lt;sub&gt;46&lt;/sub&gt;/Y121F/T289A</td>
<td>0.5</td>
</tr>
<tr>
<td>TR&lt;sub&gt;34&lt;/sub&gt;/Y121F/T289A</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*ITZ, itraconazole; VCZ, voriconazole; POS, posaconazole; TBZ, tebuconazole.

Figure 2. Allele sharing distance matrices of the microsatellite genotypes of the clinical and environmental resistant TR<sub>46</sub>/Y121F/T289A isolates, compared with TR<sub>34</sub>/L98H and wild type controls. Red dots, TR<sub>46</sub>/Y121F/T289A isolates from clinical origin; red squares, TR<sub>46</sub>/Y121F/T289A isolates from environmental origin; blue dots, TR<sub>34</sub>/L98H isolates; green dots, wild type isolates.
Clinical characteristics

Among the 15 patients identified with a TR$_{46}$/Y121F/T289A isolate, eight were diagnosed with azole-resistant invasive aspergillosis (Table 1). Three of these patients were classified as probable disease and four as proven. One patient could not be classified according to the EORTC/MSG consensus definitions [29]. This patient showed bone destruction of the skull on CT scan and \textit{A. fumigatus} was recovered repeatedly from the ear, without any other explanation. All patients with invasive aspergillosis due to TR$_{46}$/Y121F/T289A were azole-naive, except one patient with probable and one patient with proven invasive aspergillosis. At 12 weeks after recovery of the TR$_{46}$/Y121F/T289A isolate four patients had died, and two patients had persisting infection. All patients who died had received primary therapy with voriconazole, while the only two patients who survived received primary therapy with liposomal amphotericin B (Table 1).

Environmental sampling

A total of 140 azole-resistant \textit{A. fumigatus} colonies were identified, recovered from 21 locations at 9 different sites (outdoor and indoor). From three samples (entrance of one of the hospitals and two domestic homes) \textit{A. fumigatus} colonies could not be recovered due to abundant growth of zygomycetes. Analysis of the \textit{cyp51A} gene and the promoter region showed that 126 (90\%) isolates harbored TR$_{34}$/L98H, while 14 (10\%) harbored the new TR$_{46}$/Y121F/T289A resistance mechanism. Both resistance mechanisms were found in the Nijmegen and Groningen area. In 6 out of 10 sampled sites the TR$_{46}$/Y121F/T289A resistance mechanism was found, while TR$_{34}$/L98H was recovered from 9 of 10 sites (Table 4). The genotypes of 11 out of the 14 environmental TR$_{46}$/Y121F/T289A isolates 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).
Table 4. Recovery of azole-resistant *A. fumigatus* isolates through environmental air-sampling of 14,000 litre per location.

<table>
<thead>
<tr>
<th>Site</th>
<th>City</th>
<th>Location</th>
<th>Number of resistant colonies</th>
<th>TR&lt;sub&gt;34&lt;/sub&gt;/Y121F/T289A</th>
<th>TR&lt;sub&gt;34&lt;/sub&gt;/L98H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nijmegen</td>
<td>Hospital restaurant</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<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>Hospital outside</td>
<td>5</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Schaijk</td>
<td>Living room</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basement</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Back yard</td>
<td>7</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Schaijk</td>
<td>Living room</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Conservatory</td>
<td>5</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Back yard</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Overasselt</td>
<td>Kitchen</td>
<td>6</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basement</td>
<td>7</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Back yard</td>
<td>7</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Schaijk</td>
<td>Living room</td>
<td>6</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basement</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Back yard</td>
<td>3</td>
<td>-</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>3</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Balcony</td>
<td>6</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>Groningen</td>
<td>Entrance hospital*</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>
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<td>6</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>Garmerwolde</td>
<td>Living room*</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>

*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.
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 1998 [4,6]. A Dutch survey, performed between 2007 and 2009, showed that TR$_{34}$/L98H was widespread and that the prevalence varied between 0.8 and 9.5% [7]. TR$_{34}$/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 (TR$_{53}$) without mutations in the *cyp51A* gene. TR$_{53}$ was associated with a pan-azole resistant phenotype and was reported to have caused *Aspergillus* osteomyelitis in a paediatric patient in 2006 [30]. Although a TR$_{53}$ isolate was recovered from the environment (unpublished observations), there is currently no evidence for migration of this resistance mechanism.

Our current study describes TR$_{46}$/Y121F/T289A as the third resistance mechanism that has emerged in clinical and environmental isolates. Similar to TR$_{34}$/L98H [11], the fungicide-driven route of resistance development could have caused the emergence of TR$_{46}$/Y121F/T289A. Both resistance mechanisms consist of a combination of genomic changes that include a tandem repeat [15,28]. The new resistance mechanism included three 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 [14,25]. Both TR$_{34}$/L98H and TR$_{46}$/Y121F/T289A were recovered from epidemiologically unrelated patients, most of which were azole-naïve and both resistance mechanisms were recovered from the environment. Furthermore, genetic typing showed clustering of TR$_{34}$/L98H and TR$_{46}$/Y121F/T289A in separate clades apart from wild type isolates.

The evolving epidemiology of TR$_{34}$/L98H indicates that this resistance mechanism is not restricted to the Netherlands, but is increasingly being observed in other European member states [6,13-19], and outside Europe [20,21]. Genotyping indicates that TR$_{34}$/L98H isolates represent offspring of a common ancestor [22], and could have developed locally, possibly in the Netherlands, and subsequently spread across countries through wind dispersed conidia or ascospores. Given the rapid migration of TR$_{46}$/Y121F/T289A in Dutch hospitals, it can be
anticipated that this resistance mechanism will spread, similar to TR$_{34}$/L98H.

Resistance threatens the outcome of patients with *Aspergillus* diseases, especially those with azole-resistant invasive aspergillosis. Nearly 80% of TR$_{34}$/L98H isolates were resistant to voriconazole and in patients with azole-resistant invasive aspergillosis the case-fatality rate was 88% [7]. Voriconazole, which is recommended for the primary therapy of invasive aspergillosis, was uniformly inactive against TR$_{46}$/Y121F/T289A isolates and all patients with proven or probable invasive aspergillosis that had received primary therapy with voriconazole failed. 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.

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. Our surveillance network is limited by the fact that screening is performed with cultures and therefore culture-negative azole-resistant cases will not be detected. Furthermore, there is no control group of patients with azole-susceptible *Aspergillus* disease. A case-control design would help to quantify the implications of azole resistance with respect to treatment outcome and might identify risk factors.

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 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.

**Acknowledgements**

We thank P.G.H. van Kreij and L.A.M. Aarts for support in environmental sampling, all persons who gave permission for air-sampling in their homes and M.L. Langenberg for support in data collection.
References


Voriconazole resistance from clinical and environmental origin


Voriconazole resistance from clinical and environmental origin
Germinating *Neosartorya fumigata* (teleomorph of *A. fumigatus*) ascospore on Sabouraud agar (picture: Ton Rijs and Simone Camps).
General Discussion
Fungi are vastly underestimated as a problem of high disease impact for plants and animals. Fungal infections are emerging and current data indicate that fungi pose an increasing threat to plant and animal biodiversity [1]. This might be facilitated by high host- and environmental adaptation capacities of fungi. One example of environmental adaptation, described in this thesis, is the emergence of azole resistance in *A. fumigatus*, which is an increasingly important and threatening problem in the treatment of *Aspergillus* diseases.

*A. fumigatus* may follow two routes of resistance development: (i) during medical treatment of individual cases and (ii) through exposure to fungicides in the environment. Induction of resistance during medical treatment involves infection of the patient with an azole-susceptible *A. fumigatus* isolate that becomes resistant during therapy. This reflects in principle only individual patients. It is not expected that these resistant isolates will further spread, because a high diversity of resistance mechanisms has been found in individual patients. Furthermore patient-to-patient transmission is, if ever, extremely rare. The environmental route of resistance development or selection concerns exposure of *A. fumigatus* to azole fungicides, which are widely used in our environment. The widespread and intensive use of azole fungicides might induce resistance or select for resistant *A. fumigatus* isolates, and might create a selective pressure and optimal environment for resistant isolates to grow and spread. It is unknown whether azoles itself are mutagenic, thereby having the capacity to induce resistance. Another possibility is that other (environmental) factors such as ultraviolet light induce azole resistance by causing genetic alterations. In the latter situation, environmental resistance mechanisms such as TR$_{34}$/L98H, might have pre-existed in minute amounts in the population even before fungicides were applied. The mutations would then confer no advantage to the growth or survival of *A. fumigatus*, hence it would remain at a very low frequency. Once fungicides were applied, isolates harboring the resistance mechanism had a selective advantage (‘the survival of the fittest’) [2] and a building up of resistance would occur after repeated fungicide applications. Interestingly, evidence is now accumulating that antimicrobial resistance might also be predating human use of antibiotics in some cases [3]. Thus, not only ‘induction’ is a possible cause of resistance, but also ‘selection’ (of pre-existing variants) is a possibility.

**Scale of the problem**

In the year 2008, the emergence of environmental azole resistant *Aspergillus fumigatus* was already predicted [4]. Four years later, in many European countries (Austria, Belgium, Denmark, France, Germany, Italy, The Netherlands, Norway, Spain and the United Kingdom) and in Asia (China, Japan, Iran) azole resistance through the environmental route has been
described [4-13]. The prevalence of azole resistance in Nijmegen, The Netherlands increased from 0 to almost 8% within a period of 10 years [14] which is relatively fast on evolutionary time scales. What will happen if we do nothing? I expect that azole resistant A. fumigatus continues to spread globally and that the prevalence might increase further. Furthermore, more environmental resistance mechanisms will emerge and spread all over the world, such as the Cyp51A TR46/Y121F/T289A alterations described in this thesis. If the trend continues, the prevalence of azole resistance will increase to at least 50% within the years to come. A recent report describes that the overall mean burden estimate of all forms of aspergillosis is approximately 2,400,000 affected individuals annually (only in Europe) [15]. If spread continues and prevalence further increases, azole resistance will be highly problematic for millions of people around the world.

To further discuss resistance, different aspects need to be examined in detail. Therefore, I will address the problem of azole resistance in A. fumigatus from different perspectives.

**If I were a patient**

Resistance is associated with treatment failure [4,6,16-18] and animal studies show that the efficacy of azoles is reduced in mice infected with resistant isolates [19-20]. The current treatment options for patients with azole resistant aspergillosis are limited but might include an increase of drug exposure, the application of combination therapy, or non-azole class drugs. If I were a patient, I would say that it is of key importance to investigate these options without further delay to treat patients with infections caused by azole resistant A. fumigatus effectively. In addition, the possibilities of preventive measures for risk groups (i.e. vaccination) should be investigated. Furthermore, I would like to have a more personalized therapy based on my underlying immune status, and the resistance mechanism (susceptibility phenotype) of the A. fumigatus isolate causing the infection.

Besides investigating alternative treatment strategies, another important pillar in treating fungal infections is early detection of infection. I would like to be monitored for resistance such that treatment might be changed as rapid as possible, if required. Culture and subsequent in vitro susceptibility testing methods are very time consuming, although screening methods using azole-containing agars can shorten the laboratory turnaround time [21]. Another disadvantage of monitoring by classic culturing methods is a high culture negativity. Therefore, molecular tools might be used to detect resistance mutations directly in biological samples. These methods should be further developed and validated.

**If I owned a pharmaceutical company**

Azoles are the major drug class in the management of Aspergillus diseases. Current treatment options for patients suffering from azole resistant A. fumigatus disease are limited, and there are no drugs in phase III development that act on a new target. There is one new triazole
in phase III development (isavuconazole) [22], but it is not active against azole-resistant *A. fumigatus in vitro* [12]. *In vivo* models of azole-resistant infection are awaited to establish if this drug might be useful as treatment for patients with azole resistant *Aspergillus* diseases. A new intravenous formulation of posaconazole is underway which is believed to overcome the problems of exposure with the current oral solution. As posaconazole shows an attenuated activity against most *A. fumigatus* isolates that harbor a *cyp51A*-mediated resistance mechanism, increasing exposure in patients with azole resistant disease might be an option. Recent *in vivo* experimental data suggest that liposomal amphotericin B is effective irrespective of the presence of an azole resistance mechanism [23]. The efficacy of this drug in the treatment of central nervous system infection however is lower than that of voriconazole [24].

Discovery of new pathogen-specific targets is very complicated, as fungi are metabolically similar to mammalian cells. However, disease burden of invasive aspergillosis is high and new antifungal agents are required. If I were the owner of a pharmaceutical company, I would test newly developed azoles, antifungals with other targets, new formulations of current drugs, antibody-based therapeutics or prophylactic vaccinations [25] for their activity against (resistant) *A. fumigatus*.

**If I were a farmer**

Although there is accumulating evidence supporting the hypothesis that resistance has evolved in the environment under the selective pressure of azole fungicides, the link is not yet fully proven [15]. As a farmer I need fungicide treatment for maintaining healthy crops and reliable, high-quality yields [2]. According to scientific research, the five fungicides cross-reacting with medical azoles are bromuconazole, difenoconazole, epoxiconazole, propiconazole and tebuconazole. Bromuconazole is currently not permitted for use in The Netherlands [personal communication P. van der Haas, Mertens B.V. Horst, The Netherlands] so this agent does not represent a danger nowadays. Simply prohibiting the use of the other four fungicides is not feasible, as this might result in crop disease epidemics and subsequent food shortages and economic losses. However, in some cases I can choose for another antifungal. For example, I can apply prothioconazole instead of epoxiconazole or propiconazole. Difenoconazole and tebuconazole are used both singly and in combination with other fungicides [personal communication P. van der Haas, Mertens B.V. Horst, The Netherlands]. Maybe I should use them only in mixtures with effective multi-site or other non-cross-resistant fungicides to prevent survival of resistant *A. fumigatus*, but unfortunately this is not cost-effective. In the meantime, an analysis should be made to investigate whether other azole fungicides or fungicides of a different targeting class could be used in non-medical applications, with the purpose to keep the medical azoles effective for treatment of patients.
If I were a policy maker

During the last years, azole resistance in *A. fumigatus* gained some political attention. The Dutch National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu, RIVM) yearly publishes a report ‘State of Infectious Diseases in the Netherlands’. This report describes the current insight into developments of infectious diseases in the Dutch population and it is compiled for policymakers at the Ministry of Health, Welfare and Sport (Ministerie van Volksgezondheid, Welvaart en Sport, VWS). The report of the year 2009 described for the first time the emergence of azole resistance in *A. fumigatus*. Moreover, the report warned for a further increase and stated that treatment options of the life threatening infections would then be substantially limited [26]. In the year 2010, the Dutch politician Marianne Thieme addressed several questions to the Ministry of Housing, Spatial Planning and the Environment (Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer, VROM) and to the Ministry of Agriculture, Nature and Food Quality (Landbouw, Natuur en Voedselkwaliteit, LNV), regarding the harmful effects of resistant fungi in hospitals [27]. Furthermore, in the same year the Bureau Risk Assessment and Research Programming (Bureau Risicobeoordeling en onderzoeksprogrammering, BuRO) presented their risk assessment to the Ministries of LNV, VROM, and VWS. It was concluded that current knowledge is limited and that additional research needs to be performed before measures can be taken to prevent further increase and spread of resistance. The same conclusions were drawn by experts of the European Centre for Disease Prevention and Control (ECDC), who held two meetings to discuss the increasing problem of azole resistance in *A. fumigatus* (in April 2011 and January 2012).

Unfortunately, a few years after the first warning signs emerged, still little is known about the impact of azole resistant *A. fumigatus*. If I were a policy maker, I would say that it would be justified to direct more funds into the research of azole resistant aspergillosis. There is an urgent need for in-depth understanding of azole resistance to provide an improved knowledge base for governmental policy making. The lack of funding and the lack of research reminds me of the Q-fever outbreak, where the Dutch government also acted very restraint and slow, and the problem was not treated with high priority [28]. The problem of azole resistance also reminds me of the overuse of antibiotics in animal husbandry, which is known to have caused resistance in the human population.

I would emphasize that international surveillance studies and extensive programs of resistance monitoring into the field are warranted to investigate the size of the emerging problem of azole resistance more precisely. Possibly, geographic variations in the occurrence of resistance might be found. Regions with the highest incidence of resistance (‘hot spots’) might indicate the most intensive use of the at-risk fungicide(s) [29]. It needs to be analyzed what the effect will be in case the use of the five fungicides that had a high level of cross resistance is discontinued or reduced. It should be investigated whether it will result in a
decrease of the proportion of resistant *A. fumigatus* present in our environment. For example, field competition studies using various proportions of susceptible and resistant isolates could be used to observe the effect with and without fungicide pressure. Although limiting the use of the five azole fungicides might prevent additional resistance mechanisms to emerge, this might not be enough to stop resistance selection. Additional fungicides might cause cross-resistance to other *Aspergillus* species or other medically important fungi present in our environment. Therefore, monitoring should not be limited to *A. fumigatus*. In addition, non-agricultural studies are also warranted because azoles are also widely applied in industry. When an increased knowledge about azole resistance in *A. fumigatus* is obtained, we, as policy makers, should discuss how to perform effective prevention and control. Obviously, policy making should not be restricted to the Netherlands, but should be at European scale or even worldwide.

**If I were a postdoc**

The emerging problem of azole resistance in *Aspergillus fumigatus* and the postulated relationship with azole fungicide use has been met with skepticism and disbelief by physicians and researchers [30]. Obtaining research funding is problematic, as applications for funding have been refused because some reviewers do not endorse the scale and importance of the problem.

If I were a postdoc, I would like to investigate all the above mentioned research questions. But further research should not be limited to analyzing the effect of fungicide use, determining optimal treatment strategies, and policy making. It should also be aimed at understanding the exact conditions under which resistance mechanisms develop. Are azoles itself mutagenic or do other (environmental) factors play an important role in resistance development?

During medical treatment, azole resistance emerged in patients with chronic pulmonary aspergillosis and pulmonary aspergilloma, but not in patients with acute invasive aspergillosis [31]. Probably, in the case of aspergilloma or cavitary *Aspergillus* disease, the fungus is able to undergo multiple generations by the asexual way of reproduction. Sporulation (in the lung) may be important to facilitate the expression of the azole resistant phenotype, as opposed to hyphal growth which is typically found in acute invasive aspergillosis [32]. Possibly, not the asexual, but the sexual cycle is essential for the environmental developed resistance. Sexual reproduction would be expected to maintain genetic variation within populations, with the constant opportunity to generate novel genotypes. This is critical to allow response to environmental change, enable long-term survival and evolution of the species, and might produce offspring with increased resistance to antifungal agents [33-34]. It was proposed before that sexual recombination would promote a pathogen’s ability to evolve quickly in response to fungicides [35] and sexual crossing between isolates of the phytopathogen *Tapesia yallundae* has been shown to result in progeny with markedly increased resistance to
The *A. fumigatus* ‘environmental’ resistance mechanisms are complex as they contain at least two genomic changes, including the insertion of a tandem repeat in the *cyp51A* promoter region (TR\textsubscript{34}, TR\textsubscript{46}, or TR\textsubscript{53}). A resistance mechanism involving more than one genomic alteration could develop through multiple sequential events or through a single event. For example, in the case of TR\textsubscript{34}/L98H either the 34 basepair tandem repeat or the L98H substitution was introduced first, and then through continued evolution the other alteration was induced, or intragenic recombination occurred among the two pre-existing variants (TR\textsubscript{34} and L98H) already present. The latter situation was found in *M. graminicola*, where combinations of *cyp51* mutations evolved through intragenic recombination and subsequently reached high frequencies due to reduced azole sensitivity compared with the parental genotypes [37]. For that to occur, recombination (e.g. by the sexual cycle) is a prerequisite.

It appears that, in contrast to asexual reproduction, more specific conditions are needed to induce sexual reproduction in *A. fumigatus*. In the laboratory, specific nutrients and temperature were required, as well as a lot of patience as cleistothecia developed only after weeks or months of incubation. These observations do suggest that the sexual cycle may be an infrequent event (this has also been suggested by others [38]), at least compared to the abundant production of conidia by the relatively rapid asexual cycle. This might explain why only three environmental resistance mechanisms have been found (i.e. TR\textsubscript{34}/L98H, TR\textsubscript{46}/Y121F/T289A, and TR\textsubscript{53}) if the sexual cycle is indeed involved in the origin of these mechanisms.

Because asexual reproduction is extremely efficient in *A. fumigatus*, with lower metabolic costs, the ability to reproduce under a wider range of conditions and within a shorter period of time, the resistance trait would subsequently be able to migrate, for example through wind-dispersed conidia. Fungicides will provide selection pressure for the resistant genotype to persist and increase in frequency. This phenomenon has also been observed in plant pathogens, where resistant genotypes are able to rapidly increase in frequency due to strong fungicide selection coupled with an absence of fitness costs associated with the mutation [35].

Evidence to support the hypothesis that sexual reproduction plays a role in resistance development may be provided by induction experiments during sexual reproduction. For that, the actual sexual cycle should take place using pressure of the five azole fungicides that showed *in vitro* cross resistance and exhibited similar molecule structure to the medical triazoles.

Furthermore, I would sample in the environment for the sexual state of *A. fumigatus* (ascospores) with the purpose to identify the ecological niche in which it develops. Possibly, there are specific conditions in the Netherlands that facilitate the selection of azole resistance during sexual reproduction. Identification of these conditions would be informative to develop effective limiting- and preventive measures.
Chapter 7

References


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General discussion
Microscopic image of squashed cleistothecia with released asci and ascospores. In addition, hyphal fragments and asexual spores can be observed (picture: Ton Rijs and Simone Camps).
Chapter 8

Summary / Samenvatting
Summary

*Aspergillus fumigatus* is the most prevalent airborne fungal pathogen, and causes severe and often fatal invasive infections in immunocompromised hosts. Additionally, *A. fumigatus* is also able to cause a wide range of non-invasive diseases including allergic syndromes and aspergilloma. Azoles are the largest and most widely used class of antifungal drugs to treat infections caused by *A. fumigatus*. Although *A. fumigatus* is generally susceptible to these antifungals, acquired resistance is increasingly being reported over the last few years. In this thesis new mechanisms of resistance in *A. fumigatus* were discovered and investigated. The thesis consists of two sections. Section I focuses on individual cases of azole resistance during medical treatment (the clinical route of resistance) and contains chapters 2 and 3. Section II comprises chapters 4, 5 and 6 and addresses the emergence of resistance through a possible environmental route (chapter 1).

Section I. The clinical route of resistance development

Patients who are infected by an azole-susceptible isolate may develop azole-resistant disease during (chronic) azole therapy. During the course of disease, *A. fumigatus* may acquire one or more mutations in the *cyp51A* gene, which encodes the target for azole molecules. A number of mutations in *cyp51A* have been identified that have been shown to be associated with resistance. These include, for example, mutations resulting in substitutions at codons G54, M220, and G448. However, not all resistant isolates have mutations in their *cyp51A* gene, indicating that other yet unknown mechanisms might also be the cause of resistance.

In chapter 2, a patient is described in which multiple resistance mechanisms were induced during azole therapy. In this study, nine sequential *A. fumigatus* isolates were obtained from a patient with aspergilloma. Although the first isolate showed wildtype azole susceptibility, the other isolates were all resistant to itraconazole with a minimum inhibitory concentration (MIC) of >16 mg/l. The voriconazole MICs varied between 0.5 and 8 mg/l, and posaconazole MICs were between 0.5 and >16 mg/l.

Because the most common mechanisms of resistance in *A. fumigatus* are mutations in the *cyp51A* gene, this gene was sequenced in all isolates. One of the isolates had the G54E substitution which was proved to be associated with azole resistance before. The other isolates had either a P216L or F219I substitution in Cyp51A. Residues P216 and F219 both appeared to be very conserved among fungal species, indicating that these positions are important. A Cyp51A homology model based on the *Mycobacterium tuberculosis* crystal structure of the enzyme subsequently showed that P216 as well as F219 were located close to the opening of one of the ligand access channels. It is believed that through these channels, azoles dock to the heme centre of the Cyp51A enzyme, thereby blocking its function. This indicates that
both P216 and F219 residues are important and might play a role in azole resistance by blocking the entrance of azoles into the heme centre. The final proof was provided by the construction of recombinant A. fumigatus, in which the wildtype cyp51A was exchanged with a cyp51A cassette containing either one of the two mutations. Indeed, recombinants showed resistance as was observed in the patient isolates. However, five patient isolates with the F219I substitution probably continued to evolve further resistance as the voriconazole MIC further increased from 1 to 8 mg/l and the posaconazole MIC increased from 0.5 to >16 mg/l. Because no additional cyp51A mutations were found, an additional but unknown mechanism must have evolved in these isolates.

A review of the literature yielded seven other cases with proven acquired azole resistance caused by A. fumigatus. As might be expected, all patients had azole exposure before the identification of the first resistant isolate. The median time between the last cultured wildtype isolate and the first azole resistant strain was only 4 months, indicating that resistance can be induced relatively soon after initiating treatment. Furthermore, as also observed in our patient, the emergence of multiple mechanisms of resistance was common. Cyp51A mutations were observed in most but not all resistant isolates. The isolates of one of the reviewed patients are further investigated in chapter 3.

This patient suffered from chronic granulomatous disease and had a pulmonary infection caused by A. fumigatus. Four sequential isogenic A. fumigatus isolates were obtained from his respiratory samples and he was treated with multiple courses of antifungal therapy. In vitro susceptibility testing showed that the first two isolates were azole susceptible, while the last two isolates were resistant to itraconazole and voriconazole and had an intermediate susceptibility to posaconazole. Because no alterations were found in the cyp51A gene, a new, unknown resistance mechanism must have emerged during therapy.

To identify the resistance mechanism, the second and third isolate (isolated directly before and after the onset of azole resistance) were subjected to whole genome sequencing. Six non-synonymous mutations were found in the resistant isolate compared to the susceptible isolate. To elucidate which of the six mutations was the cause of resistance, sexual crosses were performed. For that, the resistant isolate was crossed with a susceptible A. fumigatus isolate of the opposite mating type. The P88L substitution in HapE was found in all resistant progeny, but never in the susceptible progeny, strongly indicating that this mutation was actually causing the resistant phenotype. Subsequent cloning of the mutated hapE gene into susceptible A. fumigatus showed that P88L in HapE could indeed confer the resistant phenotype.

HapE is a transcription factor subunit and the substitution is located in the evolutionary conserved core domain, suggesting that P88 is an important amino acid for the function of the protein. We hypothesize that the mutated Hap-complex might bind to a CCAAT-box in the promoter region of cyp51A, thereby inducing the expression of the gene and subsequently
leading to azole resistance. Alternatively, Hap might function as a transcriptional repressor, and the HapE P88L substitution could de-repress cyp51A expression. Indeed, cyp51A expression level of the resistant patient isolates was higher compared to the susceptible isolates, and a CCAAT-sequence is present in the cyp51A promoter. Importantly, the P88L substitution in HapE is the first non-cyp51A resistance mutation found in clinical A. fumigatus isolates to date.

Section II. The environmental route of resistance
Although still controversial, we hypothesize that in addition to development of resistance in azole-treated patients, resistance could also develop (or being selected for) in the environment. Several points are in favor of an environmental route, for example the finding of a dominant resistance mechanism (TR34/L98H in cyp51A) that was found in over 90% of resistant clinical isolates from different hospitals in The Netherlands. As described in the first section, in epidemiologically unlinked patients many resistance mechanisms would be expected. Furthermore, the TR34/L98H genotype was also found in azole-naïve patients, strongly indicating that resistance does not exclusively develop duringazole therapy in the patient. The finding of A. fumigatus isolates of the TR34/L98H genotype in the environment, which cluster together with clinical isolates, indicate genetic relatedness and possibly a common ancestor. Fungicides commonly used in the environment, for example those used in agriculture, could be the cause of the widespread occurrence of resistance in the environment. In chapter 4 the results further arguing for an environmental route of resistance development/selection in A. fumigatus are described. Thirty-one compounds that have been authorized for use as fungicides, herbicides, herbicide safeners and plant growth regulators were investigated for cross-resistance to medical triazoles. Of these, five triazole demethylation inhibitors (DMIs), namely bromuconazole, difenoconazole, epoxiconazole, propiconazole, and tebuconazole, showed the highest degree of cross-resistance to the medical azoles. In addition, Cyp51A homology modeling and molecule alignment studies were performed and these five DMIs showed very similar molecule structures to the medical triazoles and also adopted similar poses while docking the protein. These DMIs were authorized for use between 1990 and 1996, directly preceding the recovery of the first clinical TR34/L98H isolate in 1998. By studying molecular evolution it was calculated that the first TR34/L98H isolate would have arisen in 1997. Finally, induction experiments were performed to investigate if TR34/L98H could be induced under fungicide pressure. One isolate evolved from two copies of the tandem repeat (TR34) to three, indicating that fungicide pressure can indeed result in these genomic changes. In chapter 5 the molecular epidemiology of resistance through the TR34/L98H resistance mechanism is investigated. For that, 142 isolates were studied by analyzing several genetic markers. Results of microsatellite analysis showed that isolates with the TR34/L98H azole resistance mechanism originating from The Netherlands and seven other European countries
are genetically less diverse than azole susceptible wildtype isolates and isolates bearing other forms of azole resistance. Furthermore, Dutch isolates with the TR<sub>34</sub>/L98H genotype as well as the majority of the European isolates carrying this resistance mechanism grouped to the same three CSP types. Sexual crossing experiments demonstrated that TR<sub>34</sub>/L98H isolates are fertile and could outcross with azole-susceptible isolates of different genetic backgrounds, indicating that TR<sub>34</sub>/L98H isolates can undergo the sexual cycle in nature. Considering the results of this chapter as a whole, it is possible to speculate about the evolutionary origin and spread of the TR<sub>34</sub>/L98H azole resistance genotype within Europe. The relatively close genetic relationships and limited CSP-type diversity of the TR<sub>34</sub>/L98H isolates, as compared to azole susceptible wildtype isolates indicates that the independent and repeated emergence of the TR<sub>34</sub>/L98H mechanism seems unlikely. An alternative explanation might be that the TR<sub>34</sub>/L98H isolates developed from a common ancestor or restricted set of genetically-related isolates. A common origin of the TR<sub>34</sub>/L98H mechanism was also suggested in the previous chapter, in which it was calculated that the first TR<sub>34</sub>/L98H isolate would have emerged in the Netherlands in 1997.

Chapter 6 describes a new resistance mechanism which probably also emerged and spread via the environmental pathway. Between December 2009 and January 2011, 21 azole-resistant isolates of 15 patients from six different Dutch hospitals were obtained, all harboring TR<sub>46</sub>/Y121F/T289A alterations in Cyp51A. The majority of the patients had no history of previous azole exposure. All isolates were highly resistant to voriconazole with a MIC of ≥16 mg/l. Eight patients presented with invasive aspergillosis, of which six failed primary voriconazole therapy. Recombinant experiments confirmed the link between TR<sub>46</sub>/Y121F/T289A and the resistant phenotype. Furthermore, both Cyp51A-substitutions (Y121F and T289A) might be able to interfere with the docking modes of azole compounds, as shown in the Cyp51 homology model. TR<sub>46</sub>/Y121F/T289A was recovered through air sampling in hospitals as well as in domestic homes, supporting the environmental pathway. At present, the TR<sub>46</sub>/Y121F/T289A resistance mechanism has emerged in addition to TR<sub>34</sub>/L98H and the 2010 annual prevalence had increased to 7.2%, of which TR<sub>46</sub>/Y121F/T289A accounted for 1.4% of underlying resistance mechanisms. We concluded that the increasing prevalence of azole-resistance may be accelerated by the emergence of this new resistance mechanism and that emergence and migration of this new resistance mechanism might be threatening the use of primary therapy with voriconazole.

The general discussion of the thesis is represented in chapter 7. Both routes of azole resistance development/selection (i.e. during medical treatment of individual cases and through exposure to fungicides in the environment) appear to have different characteristics and induce specific resistance mechanisms. Treatment induced resistance often reflects substitutions in Cyp51A, such as at codons G54, P216, F219, M220, and G448. Other genes (such as hapE) are also involved, although less is
known about non-cyp51A resistance mechanisms. Furthermore, more than one resistance mechanism may arise, and resistance might accumulate. It is still unknown which proportion of azole-treated patients develop azole resistance and whether specific risk factors for resistance development can be identified. Furthermore, patients might be monitored for development of resistance such that treatment might be changed as quickly as possible after resistance emerged.

Resistance development/selection through the use of fungicides (the environmental route of resistance) might be even more worrisome. The continued use of triazole DMIs in our environment with activity against *A. fumigatus* will help resistance traits to sustain in the environment and will cause new resistance mechanism to emerge. The potential of global migration, as observed with the TR34/L98H resistance mechanism, indicates that fungicide-driven route of resistance development/selection will not remain a regional problem. We expect that unless we are able to implement measures that prevent environmental resistance development and spread, the clinical use of azoles will be severely compromised.
Aspergillus fumigatus is de meest voorkomende pathogene schimmel die via de lucht wordt verspreid. Deze schimmel kan ernstige en vaak fatale invasieve infecties veroorzaken bij immuungecompromitteerde patiënten. Ook kan *A. fumigatus* diverse niet-invasieve ziekten, zoals allergische beelden en aspergilloom veroorzaken. Azolen zijn de meest gebruikte klasse van antischimmelgeneesmiddelen voor de behandeling van *A. fumigatus* infecties. Ondanks het feit dat *A. fumigatus* het algemeen gevoelig is voor deze middelen, wordt resistentie in de laatste jaren steeds vaker gevonden.

In dit proefschrift worden nieuwe resistentiemechanismen in *A. fumigatus* beschreven en verder onderzocht. Het proefschrift bestaat uit twee secties. Sectie I focust op het ontstaan van azoolresistentie bij patiënten gedurende de behandeling met azolen (de klinische route van resistentie) en bevat hoofdstuk 2 en 3. Sectie II omvat hoofdstukken 4, 5 en 6 en behandelt de opkomst van resistentie die in de omgeving is ontstaan of geselecteerd (hoofdstuk 1).

**Sectie I. De klinische route van resistentievorming**


In hoofdstuk 2 wordt een patiënt beschreven waarin meerdere resistentiemechanismen zijn geïnduceerd tijdens de behandeling met azolen. In deze studie werden negen achtereenvolgende *A. fumigatus* isolaten verkregen van deze patiënt, die leed aan een aspergilloom. Ondanks dat het eerste isolaat een wildtype azoolgevoeligheid had, waren alle volgende isolaten (gekweekt ná het starten van de therapie) ongevoelig voor itraconazol met een minimale remmende concentratie (MRC) van >16 mg/l. De voriconazol MRC’s varieerden tussen de 0,5 en 8 mg/l en posaconazol MRC’s waren tussen 0,5 en >16 mg/l. Omdat de meest voorkomende mechanismen van resistentie in *A. fumigatus* mutaties in het *cyp51A* gen zijn, werd dit gen gesequencet in alle isolaten. Één van de isolaten had een G54E substitutie. Uit eerdere studies is gebleken dat deze substitutie geassocieerd is met azoolresistentie. De overige isolaten hadden een P216L of een F219I substitutie in Cyp51A. Residuen P216 en F219 bleken beide geconserveerde te zijn binnen de fungi. Dit geeft een indicatie van het belang van deze posities. Een Cyp51A homologie model gebaseerd op de *Mycobacterium tuberculosis* kristalstructuur van het enzym liet vervolgens zien dat P216...
alsmede F219 dicht bij de opening van één van de twee kanalen ligt. Door deze kanalen dringen azolen waarschijnlijk het haemcentrum van Cyp51A binnen en daardoor wordt de functie van het enzym geblokkeerd. Dit duidt erop dat zowel P216 als F219 belangrijk is en een rol in azoolresistentie zou kunnen spelen doordat de azolen nu niet meer door het kanaal kunnen. Het uiteindelijke bewijs werd geleverd door de constructie van A. fumigatus recombinanten, waarin het wildtype cyp51A uitgewisseld werd met een cyp51A cassette met daarin één van de twee mutaties. De recombinanten waren inderdaad resistent, net zoals de oorspronkelijke patiëntenisolaten. Vijf isolaten afkomstig van deze patiënt ontwikkelden nog verdere resistentie: de voriconazol MRC steeg van 1 tot 8 mg/l en de posaconazol MRC nam toe van 0.5 tot >16 mg/l. Omdat er geen andere cyp51A mutaties gevonden werden, is er een additioneel onbekend mechanisme ontstaan in deze isolaten.

Literatuurstudie leverde zeven andere gevallen op waarin bewezen verworven azoolresistentie in A. fumigatus is beschreven. Zoals verwacht hadden alle patiënten azooltherapie voorafgaande aan identificatie van het eerste resistentie isolaat. De mediane tijd tussen het laatst gekweekte wildtype isolaat en het eerste azoolresistente isolaat was maar 4 maanden, wat er op duidt dat resistentie relatief snel na de start van de behandeling kan ontstaan. Bovendien kwam de ontwikkeling van meerdere resistentiemechanismen vaak voor. Cyp51A mutaties werden geobserveerd in de meeste, maar niet in alle resistentie isolaten. De isolaten van één van de patiënten werden verder onderzocht in hoofdstuk 3.

Deze patiënt leed aan chronische granulomateuze ziekte en had een pulmonaire infectie veroorzaakt door A. fumigatus. Vier isogene A. fumigatus isolaten werden achtereenvolgens uit zijn respiratoire monsters verkregen en hij werd behandeld met diverse antifungale geneesmiddelen. In vitro gevoeligheidsbepalingen lieten zien dat de eerste twee isolaten azoolgevoelig waren, terwijl de laatste twee isolaten resistent waren voor itraconazol en voriconazol en een intermediaire gevoeligheid hadden voor posaconazol. Omdat er geen veranderingen werden gevonden in het cyp51A gen, moet er een nieuw, onbekend resistentiemechanisme zijn ontstaan tijdens de behandeling.


HapE is een transcriptiefactor en de substitutie is gesitueerd in het evolutionair geconserveerde ‘kern’ domein, wat suggereert dat P88 een belangrijk aminozuur is voor de functie van het
eiwit. We verwachten dat het gemuteerde Hap-complex aan een CCAAT-box in de promotor regio van *cyp51A* bindt en daarbij de expressie van het gen induceert. Dit leidt vervolgens tot azoolresistentie. Een andere mogelijkheid is, dat Hap als een transcriptionele repressor werkt, en dat de HapE P88L substitutie de remming van *cyp51A* expressie kan opheffen. Inderdaad was *cyp51A* expressie van de resistente patiëntenisolaten hoger dan de gevoelige isolaten, en er is een CCAAT sequentie aanwezig in de Cyp51A promotor. P88L is de eerste non-*cyp51A* resistentiemutatie gevonden in een klinisch *A. fumigatus* isolaat.

**Sectie II. De omgevingsroute van resistentie**

Wij hebben de hypothese geponeerd, dat behalve resistentie-ontwikkeling in azoolbehandelde patiënten, resistentie ook kan ontstaan in de omgeving. Verschillende factoren wijzen hierop, bijvoorbeeld de sterke dominantie van eenzelfde resistentiemechanisme: TR\textsubscript{34}/L98H in *cyp51A* werd in meer dan 90% van de resistentie-klinische isolaten uit verschillende ziekenhuizen in Nederland gevonden. Zoals al beschreven in de eerste sectie, zou men in epidemiologisch ongerelateerde patiënten een grotere diversiteit aan resistentiemechanismen verwachten. Bovendien werd het TR\textsubscript{34}/L98H genotype ook in azool-naïeve patiënten gevonden, wat er op wijst dat resistentie niet alleen tijdens azoolbehandeling in de patiënt kan ontstaan. We hebben ook duidelijke aanwijzingen gevonden dat *A. fumigatus* isolaten met het TR\textsubscript{34}/L98H genotype geïsoleerd uit patiënten en uit de omgeving genetisch verwant zijn aan elkaar en mogelijk een gemeenschappelijke voorouder hebben. Fungiciden worden veelvuldig gebruikt in het milieu, bijvoorbeeld in de landbouw. Dit zou de oorzaak kunnen zijn van het voorkomen van resistentie in het milieu. In **hoofdstuk 4** worden verdere resultaten beschreven die duiden op een omgevingsroute van resistentie in *A. fumigatus*. Eenendertig middelen die toegelaten zijn voor gebruik als fungicide, herbicide, herbicide safener en/of groeiregulator, werden getest op kruisresistentie met medische azolen. Van deze middelen waren er vijf triazol demethylatie inhibitors (DMI’s) met hoge kruisresistentie tegen de medische azolen (bromuconazol, difenoconazol, epoxiconazol, propiconazol, en tebuconazol). Deze DMI’s hadden een moleculstructuur vergelijkbaar aan de medische azolen. Het Cyp51A model liet ook zien dat de vijf middelen zich op eenzelfde manier positioneerden in het doelenzym. De DMI’s werden toegelaten voor gebruik tussen 1990 en 1996, precies voorafgaand aan de isolatie van de eerste TR\textsubscript{34}/L98H stam in 1998. Door het bestuderen van moleculaire evolutie is berekend dat het eerste TR\textsubscript{34}/L98H isolaat in 1997 ontstaan zou zijn. Inductie-experimenten werden vervolgens uitgevoerd om te onderzoeken of TR\textsubscript{34}/L98H geïnduceerd zou kunnen worden onder fungicide druk. Eén isolaat evolueerde van twee naar drie kopieën van de tandem repeat (TR\textsubscript{34}), dus fungicide druk zou deze genomische veranderingen tot gevolg kunnen hebben.

In **hoofdstuk 5** wordt de moleculaire epidemiologie van resistentie door het TR\textsubscript{34}/L98H resistentiemechanisme onderzocht. Daarvoor werden genetische markers van 142 isolaten
bestudeerd. De resultaten van microsatelliet analyse lieten zien dat isolaten met het TR\textsubscript{34}/L98H resistentiemechanisme uit Nederland en zeven andere Europese landen genetisch minder divers zijn dan azoolgevoelige wildtype isolaten en isolaten met andere vormen van resistentie. Bovendien groepeerden Nederlandse isolaten van het TR\textsubscript{34}/L98H genotype en de meerderheid van de Europese TR\textsubscript{34}/L98H isolaten in dezelfde drie CSP types. Geslachtelijke voortplanting toonde aan dat TR\textsubscript{34}/L98H isolaten fertiel zijn en dat deze isolaten kunnen paren met azoolgevoelige isolaten met diverse genetische achtergronden. Dit duidt erop dat TR\textsubscript{34}/L98H isolaten in de natuur de sexuele cyclus kunnen ondergaan. Met de resultaten van dit hoofdstuk kunnen we speculeren over de evolutionaire bron en de spreiding van het TR\textsubscript{34}/L98H azoolresistentie genotype binnen Europa. De relatief hechte genetische verwantschap en gelimiteerde diversiteit van CSP types, in vergelijking tot azoolgevoelige wildtype isolaten duiden niet op een onafhankelijk en herhaaldelijk ontstaan van het TR\textsubscript{34}/L98H mechanisme. Een alternatieve verklaring kan zijn dat de TR\textsubscript{34}/L98H isolaten uit een gemeenschappelijke voorouder of een beperkte set van genetisch gerelateerde isolaten zijn ontstaan. De gemeenschappelijke oorsprong van het TR\textsubscript{34}/L98H resistentiemechanisme is ook in het vorige hoofdstuk gesuggereerd, waarin werd berekend dat het eerste isolaat in Nederland in 1997 ontstaan zou zijn.

**Hoofdstuk 6** beschrijft de ontwikkeling van een nieuw resistentiemechanisme, dat waarschijnlijk ook via de omgevingsroute is ontstaan. Van december 2009 tot en met januari 2011 werden 21 azoolresistente isolaten van 15 patiënten uit zes verschillende Nederlandse ziekenhuizen verkregen, allen met de TR\textsubscript{46}/Y121F/T289A veranderingen in Cyp51A. De meeste patiënten hadden geen voorafgaande azoolmedicatie ontvangen. Alle isolaten waren resistent voor voriconazol met een MRC van ≥16 mg/l. Acht patiënten presenteerden zich met een invasieve aspergillarse, waarvan er zes faalden op primaire voriconazol therapie. Recombinant experimenten bevestigden de associatie tussen TR\textsubscript{46}/Y121F/T289A en het resistentefenotype. Bovendien liet het Cyp51A homologiemodel zien dat beide substituties (Y121F en T289A) mogelijk interfereren met de docking van azoolmoleculen in het enzym. Het TR\textsubscript{46}/Y121F/T289A genotype werd ook geïsoleerd door luchtbemonstering in ziekenhuizen en in huiselijke omgeving, wat een omgevingsgerelateerde weg suggereert. Het TR\textsubscript{46}/Y121F/T289A resistentiemechanisme is ontstaan naast TR\textsubscript{34}/L98H, en de prevalentie van azoolresistentie van 2010 steeg tot 7,2%; 1,4% werd veroorzaakt door TR\textsubscript{46}/Y121F/T289A. We concludeerden dat de prevalentie van azoolresistentie versneld kan toenemen door het ontstaan van dit nieuwe resistentiemechanisme, en dat het ontstaan en de verspreiding van dit mechanisme de primaire behandeling met voriconazol kan bedreigen.

De algemene discussie van dit proefschrift wordt beschreven in **hoofdstuk 7**. Beide routes van resistentie ontwikkeling/selectie (tijdens medische behandeling van individuele gevallen of door de blootstelling aan fungiciden in de omgeving) lijken verschillende karakteristieken en specifieke resistentiemechanismen te hebben.
Klinisch geïnduceerde resistentie heeft vaak betrekking op substituties in Cyp51A, zoals op codons G54, P216, F219, M220, en G448. Andere genen (zoals hapE) spelen ook een rol, maar er is minder bekend over niet-cyp51A resistentiemechanismen. Bovendien kan er meer dan één resistentiemechanisme ontstaan, en resistentie kan accumuleren. Het is nog steeds onbekend welke proportie van azoolbehandelde patiënten resistentie ontwikkelen en of specifieke risicofactoren voor resistentie-ontwikkeling geïdentificeerd kunnen worden. Patiënten zouden gevolgd kunnen worden voor ontwikkeling van resistentie, zodat behandeling zo snel mogelijk na resistentie-ontwikkeling gewijzigd kan worden.

Resistentie door het gebruik van fungiciden (de omgevingsroute van resistentie) is nog zorgwekkender. Het voortdurende gebruik van triazool DMI’s in ons milieu met activiteit tegen \( A. fumigatus \) zorgt ervoor dat resistentie behouden blijft en zal ook mogelijkheden geven voor de ontwikkeling en/of geografische verspreiding van nieuwe resistentiemechanismen. Het potentieel van globale verspreiding, zoals gezien met het TR\(_{34}\)/L98H resistentiemechanisme, wijst uit dat fungicide gedreven resistentie ontwikkeling/selectie geen regionaal probleem is. Wij verwachten dat, tenzij er effectieve maatregelen genomen worden om omgeving gerelateerde resistentie-ontwikkeling en verspreiding tegen te gaan, het klinische gebruik van azolen in de (nabije) toekomst ernstig belemmerd zal worden.
Microscopic view of *A. fumigatus* cultured on Sabouraud agar (picture: Ton Rijs and Simone Camps).
Chapter 9

Dankwoord / Curriculum Vitae / List of publications
Dankwoord

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Chapter 9

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Curriculum Vitae


Ze werkte als student bij de afdelingen Centraal Hematologisch Laboratorium en Medische Microbiologie van Universitair Medisch Centrum St. Radboud, Nijmegen en bij de afdeling plantengenetica aan de Radboud Universiteit, Nijmegen. Het afstuderen vond tijdens beide studies plaats bij de afdeling Virological R&D van Intervet International te Boxmeer, waar ze tijdens haar studies ook kortdurend werkte als analist.


Simone is getrouwd met Falco Rossel en zij hebben een dochter, Lisa, geboren op 15 april 2012.
List of publications


Camps SMT et al, Hospital point-of-use water filtration to prevent exposure to waterborne pathogens. *Manuscript in preparation*.

*contributed equally to the publication.