Relevance of heterokaryosis for adaptation and azole-resistance development in *Aspergillus fumigatus*

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Abstract

Aspergillus fumigatus causes a range of diseases in humans, some of which are characterized by fungal persistence. *A. fumigatus*, being a generalist saprotroph, may initially establish lung colonisation due to its physiological versatility and subsequently adapt through genetic changes to the human lung environment and antifungal treatments. Human lung-adapted genotypes can arise by spontaneous mutation and/or recombination and subsequent selection of the fittest genotypes. Sexual and asexual spores are considered crucial contributors to the genetic diversity and adaptive potential of aspergilli by recombination and mutation supply respectively. However, in certain *Aspergillus* diseases, such as cystic fibrosis and chronic pulmonary aspergillosis, *A. fumigatus* may not sporulate but persist as a network of fungal mycelium. During azole therapy, such mycelia may develop patient-acquired resistance and become heterokaryotic by mutations in one of the nuclei. We investigated the relevance of heterokaryosis for azole-resistance development in *A. fumigatus.* We found evidence for heterokaryosis of *A. fumigatus* in patients with chronic *Aspergillus* diseases. Mycelium from patient-tissue biopsies segregated different homokaryons, from which heterokaryons could be reconstructed. Whereas all variant homokaryons recovered from the same patient were capable of forming a heterokaryon, those from different patients were heterokaryon-incompatible. We furthermore compared heterokaryons and heterozygous diploids constructed from environmental isolates with different levels of azole resistance. When exposed to azole, the heterokaryons revealed remarkable shifts in their nuclear ratio, and the resistance level of heterokaryons exceeded that of the corresponding heterozygous diploids.

Key words: *Aspergillus fumigatus*; heterokaryon incompatibility; azole resistance; diploid; flexible nuclear ratio

Introduction

The vast majority of invasive mould infections in humans are caused by *Aspergillus fumigatus*. Azole antifungals are the mainstay of management of *Aspergillus* diseases, but treatment is hampered by the emergence of multi-azole resistant *A. fumigatus* isolates[1-3]. Azole resistance is now reported globally [4-12], with resistance rates varying between 3.9% and 19% in clinical isolates in the Netherlands [13, 14] and 20% in the National Aspergillosis Centre of the United Kingdom [15-17]. The majority of highly resistant mutants have modifications in the coding and promoter region of the *cyp51A*-gene[18-20]. This gene encodes the enzyme lanosterol 14- alpha-demethylase (CYP51A), the target of azoles that is essential to the ergosterol synthesis pathway. These *cyp51A* mutations are believed to emerge by exposure of *A. fumigatus* to medical azoles or azole fungicides [21].

Although many aspects of azole-resistance development in *A. fumigatus* remain to be investigated, the capacity of the fungus to create genetic diversity is crucial. Genetic variation may be generated during the various aspects of the lifecycle of *A. fumigatus*: mycelial growth, asexual sporulation and sexual reproduction. Several studies in *A. fumigatus* have focussed on the consequences of asexual [22] and sexual reproduction for fungal adaptation [23, 24], but the role of the mycelium has been largely overlooked. Although patient-acquired azole resistance appears to be associated with the presence of a pulmonary cavity, which is an environment that allows for asexual reproduction, *A. fumigatus* hyphal biofilms are present in patients with cystic fibrosis (CF) and chronic pulmonary aspergillosis (CPA). A variety of azole-resistance mutations has been observed in patients with CF and CPA, indicating that fungal adaptation takes place [4]. Somatic mutations may occur during mitotic divisions leading to genetic variation

within the mycelium. Such heterokaryotic mycelium may subsequently undergo parasexual recombination and segregate with a genetic variety of clones [25, 26] .

Heterokaryosis, i.e. genetically different nuclei within the same cytoplasm, is common in fungi. Hansen (1938) found that in various fungi more than 50% of natural mycelia were heterokaryons that upon single-spore culturing segregated in cultures of morphologically distinct types with respect to abundance of aerial hyphae and conidia produced [27]. A heterokaryon can result from mutations in one or more nuclei in a homokaryotic mycelium, or from anastomosis of hyphae from genetically distinct homokaryons. The latter is however restricted by heterokaryon incompatibility, a common fungal allorecognition mechanism limiting successful fusion of hyphae to clonally related strains with the same heterokaryon-compatibility alleles [28-30]. Heterokaryon incompatibility has not been demonstrated yet in *A. fumigatus*, but if it exists, mutation is the likely initial cause of heterokaryon formation in patients, especially for isolated long-lasting cultures.

Since asexual spores of *A. fumigatus* are uninucleate, newly formed colonies start as a homokaryon and may produce heterokaryon during mycelial growth. Heterokaryosis is thus a transient characteristic of the mycelium that is lost upon asexual sporulation and dispersal of spores by air. Therefore, heterokaryons may form and persist particularly in long-lived mycelium cultures. This may be the case for chronic *A. fumigatus* infections, where the fungus has been shown to persist sometimes for many years [1, 31-33]. Over time, emergence of azole resistance in chronically colonized patients has indeed been described in consecutive *A. fumigatus* cultures that were concluded to be isogenic based on microsatellite genotyping [4, 34]. Evidence for a possible role of heterokaryosis in azole-resistance development was also found in evolutionary laboratory experiments where an ancestral strain was allowed to evolve resistance during several weeks of

azole exposure [22]. Both from patients and from evolutionary laboratory experiments, different evolved clones of *A. fumigatus* have been isolated, some of which are poorly sporulating or completely aconidial [35, 36]. Different morphotypes from patients have been encountered especially in chronic infections either sinusitis, aspergilloma or in CF [6, 31, 35-37] but the characteristics and significance of such variants has not been studied.

Here, we study the relevance of heterokaryosis and somatic variation in the adaptive development of *A. fumigatus.* We address the following questions.

(I) What are the characteristics of different variants of *A. fumigatus* isolated from evolved cultures of patients or lab experiments? (II) Are the different successive or coexisting variants from a patient heterokaryon – compatible, and does heterokaryon incompatibility exist in *A. fumigatus* ? (III) How does azole resistance of heterokaryons relate to that of the individual homokaryons and heterozygous diploids? (VI) Is there plasticity in the nuclear ratio within a heterokaryon in response to changing azole concentrations?

Material and methods

Strains used in this study

Fourteen clinical *A. fumigatus* strains, stored in the Radboud University Medical Centre, were available from five different patients suffering from various *Aspergillus* diseases (see Table 1 for patient characteristics). Four successive isolates were collected from patients P1 (aspergilloma) and P2 (chronic granulomatous disease) during azole treatment, another two strains were isolated from patient P3, a kidney transplant recipient, patient P4 with invasive aspergillosis and patient P5 a CF patient.

Furthermore we used wild-type strain CBS140053 (isolated from soil in Wageningen, The Netherlands, 1992) and five derived strains, that evolved during a seven weeks evolutionary experiment on medium with 1 µg/mL difenoconazole (Zhang et al. 2015b; Table 2).

Culture media

Minimal Medium (MM) was used for culturing heterokaryon and heterozygous diploid. MM consists of 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$7. $7H_2O$, 0.5 g KCl, 10 mg of $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$ and 15 g agar + 1000 mL H_2O (pH 5.8). Malt extract agar (MEA), used for counting spores and measuring the mycelial growth rate, was purchased from Sigma Company (Sigma Aldrich, Germany). The azole fungicide difenoconazole (DIF), and the medical azoles itraconazole (ITR), voriconazole (VOR) and posaconazole (POS) were purchased from Sigma Company (Sigma Aldrich, Germany) [22].

Aspergillus fumigatus variants and morphotypes isolated from evolved cultures of patients and experimental evolution experiments

The isolates of *A. fumigatus* that were derived from the same patient or evolution experiment showed considerable variation. Morphological phenotypes were studied on MEA plates after four days incubation at 37°C. The spore size of *A. fumigatus* was measured with a Coulter counter (Beckman Coulter, the Netherlands). Mycelium Growth Rate (MGR) assays were performed by averaging the colony diameters (in mm) as measured in two randomly chosen perpendicular directions after 4 days of growth. We performed antifungal susceptibility testing and determined the minimal inhibitory concentration (MIC) for environmental isolates and clinical isolates from patients P3 and P4 according to the European Committee on Antimicrobial Susceptibility Testing

(EUCAST) reference method. Relevant clinical information from patients P1, P2 and P3 was retrieved from the literature [4, 34, 38].

Heterokaryon compatibility testing

The capacity of strains to form heterokaryon (heterokaryon compatibility) was tested following standard methods as explained in Figure 1 [39-41]. The complementing recessive nitrate non-utilizing mutations, *nia* and *cnx*, were introduced by ultra violet (UV) radiation of conidiospores of the various strains. Heterokaryon compatibility was tested among all clinical isolates from different patients and evolutionary line isolates.

Heterokaryons and diploids construction

From the evolutionary line, three environmental compatible isolates (CBS 140053-sensitive (S), CBS 140053-D3-7B- intermediate (I) and CBS 140053-D1-7- resistant (R)) with a different level of resistance to difenoconazole (see ESM Table 1) were chosen for further heterokaryon and diploid formation. Heterokaryons (S_{NG}&S_{CW}; I_{NG}&S_{CW}; R_{NG}&S_{CW}) were constructed from sensitive (S), intermediate (I) and resistant (R) strains differing in their nia or cnx mutation and conidial colour. E.g. S_{CW} is a sensitive stain with a *cnx* mutation and white spores, whereas R_{NG} is a resistant strain with a *nia* mutation and green spores. Heterozygous diploids (S_{NG}//S_{CW}; I_{NG}//S_{CW}; R_{NG}//S_{CW}) were isolated from heterokaryons by using the sandwich method [42]. MGR assays of heterokaryons and diploids were compared after 4 days of growth. Heterozygous diploids and constructed heterokaryons were inoculated on MM and MM supplemented with 1µg/ml of difenoconazole and 0.6 µg/ml of voriconazole, for individual homokaryons, MM + ureum with 1µg/ml of difenoconazole and 0.6 µg/ml of voriconazole were used.

The nuclear ratio in heterokaryons in different azole environments

Assuming that the nuclear ratio among the conidiospores reflects the nuclear ratio within the mycelium, we tested the effect of azole exposure on the constitution of the heterokaryon. In brief, heterokaryons (S_{NG}&S_{CW}; I_{NG}&S_{CW}; R_{NG}&S_{CW}) were grown on MM plates with or without 1 μg/mL difenoconazole. After 4 days of growth, spores were harvested from the heterokaryon into 0.5 mL of saline (distilled water with NaCl 0.8 g/L) supplemented with Tween 80 (0.05 % v/v) and dilutions were spread on a MEA agar plate. The number of colonies of either colour was counted and the ratio was calculated. SPSS independent samples T-test was used for statistical analyses.

Results

Different variants and morphotypes occur in successive and co-existing isolates from the same patient, and from an evolutionary experiment.

The four consecutive *A. fumigatus* strains from patient P1, and the four from patient P2 showed an increase in spore size, a decrease in growth rate and an increase in azole resistance over time (Table 2, Figure 2). The two isolates of patients P3 and P4, cultured from the same clinical specimen, were morphologically very distinct (Figure 2). Also the cultures collected in the seven weeks evolution experiment showed different morphology when compared to the ancestor and each other in addition to increasing azole resistance (Table 2, Figure 2).

Variant strains from the same patient or evolution experiment are heterokaryon compatible, strains from different patients are heterokaryon incompatible

The heterokaryon compatibility test is based on complementation of recessive deficiency markers in a heterokaryon (see Figure 1). For many fungi, including several *Aspergilli* it has been found that only clonally related isolates are heterokaryon compatible, whereas non-clonal isolates are heterokaryon incompatible. Isolates from the evolution experiments all share a common ancestor and are therefore expected to be heterokaryon compatible. We first tested these known isogenic lines from the evolutionary experiment. Indeed all these isolates, even though morphogically distinct (Figure 1), were heterokaryon compatible. This indicates that heterokaryon compatibility is stable during the evolutionary experiment. We next introduced heterokaryon-forcing markers in the isolates from patients P1, P2, P3 and P5 and tested for heterokaryon compatibility. We found that all within-patient isolates were heterokaryon compatible while isolates from different patients showed heterokaryon incompatibility (Table 3). It was not possible to obtain markers from the non-sporulating isolate from patient P4 and therefore this isolate did not allow for heterokaryon testing.

A. fumigatus heterokaryons constructed from environmental isolates have higher azole resistance than corresponding heterozygous diploids

The azole-resistance level, measured as the MGR on difenoconazole-containing medium (1µg/ml) or voriconazole medium (0.6µg/ml), of heterozygous diploids constructed from environmental isolates was compared to that of corresponding heterokaryons and their constituting haploid strains (Figure 3). The MGRs of the heterokaryons (I_{NG}&S_{CW};

206 R_{NG}&S_{CW}) were significantly higher (T- test, $T_{6,2}$ = -7.348 P < 0.05; T- test, $T_{6,2}$ = -6.791P
207 < 0.05) than those of heterozygous diploids (I_{NG}//S_{CW}; R_{NG}//S_{CW}). Also on voriconazole
208 medium (0.6μg/ml) heterokaryons showed higher MGRs than the heterozygous diploid
209 (T- test, $T_{6,2}$ = 4.866, P < 0.05; T- test, $T_{6,2}$ = 1.557, P < 0.05).

210 **The nuclear ratio in heterokaryons is flexible**

211 The growth pattern of heterokaryons was strikingly different on medium without azoles
212 compared to that on medium with 1 μg/mL difenoconazole. Whereas erratic growth of
213 the forced heterokaryon was typically seen on medium without azoles, a more compact
214 circular colony was formed on azole-containing medium (Figure 4A). The use of a white
215 spore-colour mutation in one of the strains in a heterokaryon allowed for direct testing
216 of the nuclear ratio in a heterokaryon on media with or without azoles (Figure 4B). The
217 ratios of white to green colonies from spores of heterokaryons S_{NG}&S_{CW}, I_{NG}&S_{CW} and
218 R_{NG}&S_{CW} were analysed (Figure 5). For the sensitive heterokaryon S_{CW}&S_{NG}, there was
219 no significant difference between the nuclear ratio on medium with and without 1 μg/ml
220 difenoconazole (T- test, $T_{6,2}$ = -2.151 P > 0.05). For the heterokaryon formed between
221 the sensitive and intermediately resistant nuclei (I_{NG}&S_{CW}) the percentage of
222 intermediate resistant nuclear, I_{NG} shifted from 32% to 50%: a 1.7-fold increase (T- test,
223 $T_{6,2}$ = -9.843 P < 0.01). For the heterokaryon between sensitive and resistant nuclei
224 (R_{NG}&S_{CW}), the percentage of R_{NG}-nuclei shifted from 25% to 78%: a 3.5-fold increase (T-
225 test, $T_{6,2}$ = -38.689 P < 0.01). On medium without azole, the fraction R_{NG} in R_{NG}&S_{CW}
226 heterokaryon as well as of I_{NG} in I_{NG}&S_{CW} heterokaryon was significantly lower than the
227 fraction S_{NG} in S_{NG}&S_{CW} (T- test, $T_{6,2}$ = 3.587 P < 0.05), which suggests a cost of
228 resistance of R_{NG} and I_{NG} on MM without azole (Figure 5).
229

Discussion

In this study, we investigated the relevance of heterokaryosis for *A. fumigatus* infection and persistence. We provide evidence for heterokaryosis of *A. fumigatus* isolates recovered from patients with chronic *Aspergillus* diseases. Furthermore, we demonstrate that heterokaryons constructed from environmental isolates have growth characteristics different from homokaryons and heterozygous diploids and add to the phenotypical plasticity of the isolate. The higher mycelial growth rate of a heterokaryon than the corresponding heterozygous diploids upon azole exposure is associated with a nuclear ratio shift in the heterokaryon. In addition, we formally demonstrate the existence of heterokaryon incompatibility between *A. fumigatus* isolates from different patients and an environmental strain.

A. fumigatus is a generalist saprotrophic fungus that thrives in decaying plant material, and causes, as a side effect of its metabolic versatility, opportunistic and chronic infections in various patient groups. Survival in the human host involves adaptation to the lung environment, often accompanied by development of azole resistance in patients receiving chronic azole therapy. In general, *A. fumigatus* may adapt to any new environment through genetic and phenotypic plasticity. Genetic variability is created by recombination and mutation. Recombination, at least in the laboratory, can arise during sexual reproduction, followed by selection of progeny most capable to survive [24, 43]. Mutation, the ultimate source of genetic variation, was shown to arise particularly during abundant asexual spore formation [22]. However, in the patient, sexual reproduction of *A. fumigatus* is not likely, and asexual reproduction may only occur when a pulmonary cavity is present. In addition to genetic changes, somatic variation by

heterokaryosis is another mechanism of increasing phenotypic plasticity facilitating adaptation [44].

Like heterozygosity in diploids, heterokaryosis allows genetic complementation of recessive mutations and heterosis effects. Heterokaryons are phenotypically more flexible than diploids, as in a heterokaryon all possible nuclear ratios can occur from 0 to 100 percent and ratios can change depending on environmental conditions, whereas in a heterozygous diploid the allele frequency is fixed at 50 percent. Natural heterokaryons have been described that enjoy an advantage in growth rate compared to the homokaryons under specific conditions and changing of nuclear ratios [44]. Heterokaryons can occur in many fungal species, but the biological significance remains largely unclear [27, 44, 45]. A heterokaryon can store and facilitate genetic variation by somatic mutation, parasexual recombination and segregation upon selection pressure. This aspect of fungal growth may be particularly relevant to allow persistence of mycelial forms in the lung, but has so far not received much attention in the research of *Aspergillus* infections.

Whereas colonies of *A. fumigatus* in nature that develop and reproduce within days on relatively short-lived substrates are likely ephemeral, a single inhaled uninucleate airborne conidiospore of *A. fumigatus* may establish an infection or airway colonisation that may persist as mycelium for many years in the patient [1]. During this long period, the multinucleate mycelium may enable adaptation to the host stress factors such as antifungal treatment. Several observations are consistent with derived genetic variation in *Aspergillus* species that persist in the host. When *Aspergillus* infections from patients are sampled and tested, morphologically distinct variants may be cultured in successive respiratory samples or even from the same clinical sample. These colonies might differ

277 in certain characteristics such as azole-resistance phenotype [4, 34, 35]. Particularly in
278 CF-patients *A. fumigatus* is thought to persist by the formation of biofilms and thus as
279 exclusive mycelial morphotype [46]. Microsatellite genotyping indicates that a single *A.*
280 *fumigatus* genotype may persist for many years [31-33]. Phenotypic variation in *A.*
281 *fumigatus* that is regularly found in patient cultures is also observed in laboratory
282 evolution experiments initiated with a single ancestral genotype [22], but the underlying
283 genetic background and possible clinical relevance of successive and co-isolated
284 morphotypes is unclear.

285 We here present the evidence for heterokaryosis in clinical isolates. Mycelium from
286 tissue samples of patients segregated different homokaryons, from which heterokaryons
287 could be reconstructed. All variant isolates recovered from the same patient were
288 capable of forming a heterokaryon, whereas those from different patients were
289 heterokaryon-incompatible. *A. fumigatus* heterokaryons, composed of environmental
290 homokaryons of different azole-resistance phenotypes, shifted the nuclear ratio of the
291 heterokaryon towards more of the resistant nucleus in response to azole exposure. This
292 indicates that heterokaryons of *A. fumigatus* are indeed flexible in their nuclear
293 composition and have phenotypic characteristics different from homokaryons and
294 heterozygous diploids, a process that might occur in the human lung and represent an
295 important adaptation strategy.

296 Heterokaryon can formally also arise by anastomosis of hyphae from different strains,
297 e.g. following multiple infections, but this is restricted by heterokaryon incompatibility
298 which is widespread in fungi [28, 30]. As a result of high polymorphism for
299 heterokaryon incompatibility genes (*het*-genes) in many fungal populations,
300 heterokaryon compatibility is mostly restricted to clonally related isolates and,

therefore two randomly picked fungal isolates from nature are most likely heterokaryon incompatible [47-49]. Heterokaryon incompatibility was not known in *A. fumigatus* yet, but here we clearly find evidence for its existence. We found that *A. fumigatus* isolates from different patients, and a field isolate, were heterokaryon incompatible indicating that they were all of different clonal origin. On the other hand, variant isolates recovered from the same patient that differed in growth characteristics, spore size and/or azole resistance, could form a heterokaryon, indicating a single clonal origin of the infection. Also the isogenic variant isolates derived from a common ancestor during a seven-week experimental evolution showed heterokaryon compatibility.

Relevance of heterokaryon formation for azole-resistance development and in-host adaptation

Our finding that heterokaryon compatible *A. fumigatus* variant isolates can develop in patients indicates a potential role for heterokaryosis in azole-resistance development and in-host adaptation. Patient-acquired resistance can be concluded when a phenotype switch takes place from azole-sensitive to azole-resistant in consecutive isolates recovered from individual patients. This has been observed in isogenic isolates recovered from patients treated with azoles and who have a pulmonary cavity in which asexual sporulation can occur [34].

However, azole-resistant *A. fumigatus* has also been recovered from patients without pulmonary cavities, notably patients with CF or aspergillosis [50]. In this patient group heterokaryon formation might be a strategy to develop azole resistance. Adaptive nuclear variation in a heterokaryotic *Aspergillus* mycelium network may involve component mutant nuclei that vary in level of in-host adaptation and azole resistance but together may contribute to a flexible and resilient heterokaryotic fungal infection.

Such heterokaryon will break up in single genotypes when grown on laboratory plates and may exhibit low fitness under these conditions, that can be recognized as pleiomorphic growth.

Implications of heterokaryosis in *Aspergillus* infections and future outlook

We provide evidence for heterokaryosis in clinical *A. fumigatus* isolates from patients with chronic or persistent *Aspergillus* diseases, the segregation of different homokaryons and heterokaryon compatibility. Furthermore, we show that constructed heterokaryons exhibit different growth characteristics compared with homokaryons and, at least for the environmental isolates in which it could be tested, a higher azole resistance than the corresponding heterozygous diploid. These changes were associated with a nuclear ratio change, implying a potential role for heterokaryosis in somatic variation and adaptation in patients with chronic *Aspergillus* infection and colonisation. In clinical microbiology single *Aspergillus* colonies are used to determine species identification, but little attention is paid to variability in colony morphology. In the best practice guidelines of the British Society of Medical Mycology, no mention is made of variations of colony phenotypes and the implications thereof [51]. With the emergence of azole resistance more interest was gained to analyse multiple colonies for azole-resistance testing, as mixed infection involving different azole phenotypes have been reported [52]. Also atypical colony morphology or pleiomorphic growth under lab conditions may be an indicator of in-host adaptation of the fungus, and this might have clinical relevance. In-host adaptation might reduce our therapeutic options to eradicate the fungus from the lung [53].

Further research should shed more light on the relevance of heterokaryosis in *Aspergillus* infections. How common is our finding of heterokaryon compatible

concomitant *Aspergillus* variants from different types of patients? What is the extent of heterokaryon incompatibility both in patient isolates as well as in the field? Can evidence be obtained of flexible heterokaryotic growth in patients? If so, does this contribute to azole resistance and fungal persistence in the human host? Answers to these questions may change our view on how to diagnose and manage *Aspergillus* infection and colonisation.

Conclusion

We provide evidence for heterokaryosis of *A. fumigatus* in patients with chronic *Aspergillus* diseases. Our results indicate that somatic variation and nuclear flexibility of heterokaryotic mycelium may be a thus far overlooked strategy for *A. fumigatus* to adapt to the lung environment and to overcome azole exposure. These results indicate that chronic *Aspergillus* infections are likely heterokaryotic rather than of single genotype. Implications for diagnosis and management of *A. fumigatus* infections are considered.

Competing interests

The authors declare no competing interests.

Author contributions

BJZ, PEV JZ, AJMD and SES initiated the project; JZ, AJMD and PEV designed the methodology; EJK, PEV and MCA provided materials; JZ carried out experiments and formal analyses. JZ, ES and AJMD prepared the draft of the manuscript. All authors contributed to the commenting and editing of subsequent versions of the manuscript.

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Data accessibility

All data is available on Dryad (http://datadryad.org/) with doi: 10.5061/dryad.fr3kt2d

Data files: data from the Relevance of heterokaryosis for adaptation and azole-resistance development in *Aspergillus fumigatus.*

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Tables and legends

Table 1. 5 Patient characteristics including the underlying disease, the number of isolates collected and resistance switch during the treatment.

Patient number	**Age / sex**	**Underlying disease**	***Aspergillus* disease**	**# of *A. fumigatus* isolates**	**Characteristics**	**Reference**
P1	1. 48 / M*	TNF receptor-associated periodic syndrome (TRAPS)	Aspergilloma	4	Phenotype switch from azole-susceptible to azole-resistant.	Camps 2012b
P2	2. 21 / M*	Chronic granulomatous disease	Relapse invasive aspergillosis	4	Phenotype switch from azole-susceptible to azole-resistant and fitness-cost.	Arendrup 2010, Camps 2012b
P3	3. 59 / F*	Kidney transplant	Invasive aspergillosis with abdominal abscess	2	Azole-resistant, different colony phenotypes.	Kuipers 2011
P4	4. 62 / M	Liver cirrhosis	*Aspergillus* sinusitis	2	Azole-susceptible, different colony phenotypes	This study
P5	5. 28 / F	Cystic Fibrosis (CF)	Chronic colonization since 2004	2	Azole-susceptible, cultured 27 months apart (2013 and 2015).	This study

*patient died

544 **Table 2.** Consecutive and co-existing isolates from five patients and from laboratory evolution experiments with different macroscopic
545 morphology, azole-resistance mutations and resistance phenotype.
546

Strains		Isolation date	Specimen	Treatment / exposure [1]	Spore size(µm)	Growth rate on MEA (cm/day))	Azole-resistance mutations[4]	MIC (resistance level)[2]		
								ITR	VOR	POS
P1	V74-618	29/09/2008	sputum	ITC	2.55 ± 0.02	9.375 ± 0.03	CYP51A(A9T)	0.5	1	0.063
	V77-418	17/12/2008	sputum	POS	2.65 ± 0.01	8.125 ± 0.02	CYP51A(A9T/F291I)	**>16**	1	**1**
	V80-289	09/03/2009	sputum	POS	2.65 ± 0.01	6.875 ± 0.01	CYP51A(A9T/F291I)	**>16**	**8**	**>16**
	V83-149	07/06/2009	BAL [3]	L -AMB + CAS	2.70 ± 0.01	6.625 ± 0.01	CYP51A(A9T/G54E)	**>16**	0.5	**1**
P2	V67-350	0 week	respiratory	VOR	2.50±0.02	10.25 ± 0.04	NC	0.125	0.5	0.016
	V67-361	108 weeks	respiratory	CAS+POS	2.72±0.01	8.875 ± 0.02	NC	0.25	0.5	0.031
	V67-371	125 weeks	respiratory	CAS+POS	2.87±0.02	8.375 ± 0.03	HapE(P88L)	**>16**	**4**	0.25
	V67-381	127 weeks	respiratory	CAS+POS	2.87±0.02	6.25 ± 0.01	HapE(P88L)	**>16**	**4**	0.25
P3	V094-15	20/02/2010	subcutaneous abscess	--	2.77±0.02	4.125 ± 0.02	CYP51A(TR_{46}/Y121F/T289A)	1	**>16**	0.25
	V094-16	20/02/2010	subcutaneous abscess	--	2.76±0.02	7.375 ± 0.01	CYP51A(TR_{46}/Y121F/T289A)	0.5	**>16**	0.25
P4	V186-81	03/09/2015	sfenoid	--	2.65±0.01	6.625 ± 0.01	NC	0.25	0.25	0.063

	V187-02	03/09/2015	sfenoid		--	3.75 ± 0.02	NC				
P5	V148-33	06/05/2013	sputum	no antifungal	2.52 ±0.01	3.4 ±0.01	NC	0.5	0.5	5	0.12
	V186-45	29/08/2015	sputum	no antifungal	2.52 ±0.01	3.4 ±0.01	NC	0.5	1		0.25
E1	CBS 140053	0 week	Agricultural field	--	2.49±0.02	3.125 ± 0.01	NC	0.125	0.5	6	0.01
	CBS 140053 D_1-7	7 weeks	Evolutionary lines	DIF	2.53±0.01	8.625 ± 0.03	CYP51A(G138S)	**32**	**4**		**0.5**
E3	CBS 140053 D_3-7A	7 weeks	Evolutionary lines	DIF	2.46±0.02	6.25 ± 0.03	NC	**8**	2		**0.5**
	CBS 140053 D_3-7B	7 weeks	Evolutionary lines	DIF	2.51±0.01	6.125 ± 0.01	NC	**8**	1		**0.5**
E6	CBS 140053 D_6-7A	7 weeks	Evolutionary lines	DIF	2.49±0.01	3.125 ± 0.02	NC	**8(50%)/ 16(50%)**	2		0.25
	CBS 140053 D_6-7B	7 weeks	Evolutionary lines	DIF	2.47±0.02	6.25 ± 0.01	NC	**8**	2		**0.5**

[1] CAS, caspofungin; DIF, difenoconazole; ITR, itraconazole; L-AMB: liposomal amphotericin B, ; POS, posaconazole; VOR, voriconazole

[2] MIC (Minimal Inhibitory Concentration), was determined four times for each isolate, most replicates showed consistence within replicates. There is one exception in E6, half showed 8 and another half showed 16. Resistance is indicated in bold (Clinical breakpoints for resistance are MIC > 0.25 mg/L for POS and > 2mg/L for ITR and, VOR).

[3]BAL: bronchoalveolar lavage; -- information is not available. [4]Amino acid changes in CYP51A and HapE, NC: no changes both on CYP51A and HapE.

552 Table 3. Compatibility testing of all patient strains and evolutionary evolved strains.

	P 1	P 1	P 1	P 1	P 2	P 2	P 2	P 2	P 3	P 3	P 5	P 5	E1	E1	E2	E2	E3	E3
ompatibility	V74-61	V77-41	V80-28	V83-14	V67-35	V67-36	V094-15	V094-16	V186-81	V187-02	V186-45	V148-33	CBS 140053	CBS 140053 D_1-7	CBS 140053 D3-7A	CBS 140053 D3-7B	CBS 140053 D6-7A	CBS 140053 D6-7B
74-61	C	C	C	C	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
77-41	C	C	C	C	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
80-28	C	C	C	C	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
83-14	C	C	C	C	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
67-35	NC	NC	NC	NC	C	C	C	C	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
67-36	NC	NC	NC	NC	C	C	C	C	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
094-15	NC	NC	NC	NC	C	C	C	C	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
094-16	NC	NC	NC	NC	C	C	C	C	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
186-81	NC	NC	NC	NC	NC	NC	NC	NC	C	C	NC	NC	NC	NC	NC	NC	NC	NC
187-02	NC	NC	NC	NC	NC	NC	NC	NC	C	C	NC	NC	NC	NC	NC	NC	NC	NC
186-45	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	C	C	NC	NC	NC	NC	NC	NC
148-33	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	C	C	NC	NC	NC	NC	NC	NC
BS 140053	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	C	C	C	C	C	C
BS 140053 D_1-7	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	C	C	C	C	C	C
BS 140053 3-7A	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	C	C	C	C	C	C
BS 140053 3-7B	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	C	C	C	C	C	C
BS 140053 6-7A	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	C	C	C	C	C	C
BS 140053 6-7B	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	C	C	C	C	C	C

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554 P: patient isolate; E: evolutionary line; C: compatible; NC: not compatible. From patient 4 no mutants were derived and therefore no heterokaryon compatibility could be tested.

555 ## Figures and legends

556 **Figure 1.** Different morphotypes among consecutive and co-existing isolates from
557 patients (P1 – P5) as well as from laboratory evolution experiments (E1 – E3) (see also
558 Table 1).

559 **Figure 2**. Heterokaryon compatibility test. *A. fumigatus* strains with complementing
560 mutations in different genes required for nitrate utilisation (*nia* and *cnx*) are inoculated
561 separately on the left- or right-hand side and together in the middle of the agar plate
562 with nitrate as the sole N-source (positions a and b). **A.** Heterokaryon formation
563 between co-inoculated heterokaryon-compatible strains results in complementation and
564 vigorous growth on nitrate as sole N-source after 7 days at 37^{o} C. **B.** Strains that are
565 heterokaryon incompatible do not show complementation.

566 **Figure 3**. The azole resistance of heterokaryons constructed from environmental
567 isolates relative to that of the individual homokaryons and heterozygous diploids was
568 measured as the mycelial growth rate on azole (1µg/ml of difenoconazole and 0.6 µg/ml
569 of voriconazole)-containing media (For diploid and heterokaryon, MM with azole was
570 used; for individual homokaryons, MM + ure with azole was used). R//S and I//S are
571 diploids; R&S and I&S are heterokaryons.

572 **Figure 4**. Heterokaryon S_{CW} & R_{NG} (see supplement 1) growing on heterokaryon-
573 selective medium with (A) difenoconazole (1µg/ml). B: Spore sample from the
574 heterokaryon plated on non-selective medium shows segregation into homokaryotic
575 white-spored sensitive and green-spored resistant cultures.

576 **Figure 5**. The nuclear ratio of heterokaryons (S_{NG} & S_{CW}, I_{NG} & S_{CW} and R_{NG} & S_{CW}) based
577 on the nuclear ratio in the uni-nucleate spores from the heterokaryon grown on
578 different concentrations of difenoconazole (0 or 1µg/mL).