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Narrative review

Triazole resistance in *Aspergillus fumigatus*: recent insights and challenges for patient management

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ABSTRACT

Background: Triazole resistance in *Aspergillus fumigatus* is widespread and threatens first-line triazole therapy in patients with *Aspergillus* diseases.
Objectives: To give an overview of the microbiology, epidemiology and clinical significance of triazole resistance in aspergillosis.
Sources: PubMed search for articles on resistance in *Aspergillus* species.
Content: Triazoles are not mutagenic but select resistance when spontaneous mutations occur that are better able to proliferate in the triazole-containing environment. The major target for resistance mutations involves the *Cyp51A* gene, encoding an enzyme involved in cell wall synthesis. Triazole-resistance selection environments include patient treatment and organic matter containing triazole fungicide residues. Reported resistance frequencies vary widely between countries and hospitals, and resistance significantly complicates the diagnosis and treatment of *Aspergillus* diseases. Cultures may harbour various resistance phenotypes and multiple colonies must be analysed to detect resistance. PCR tests have become available for resistance detection in culture-negative patients, but show limited sensitivity. Individuals with triazole-resistant invasive aspergillosis have a 21% higher day-42 mortality compared with triazole-susceptible infection, and to prevent excess mortality resistant cases require first-line therapy that covers resistance. The recent ESCMID-ECMM-ERS *Aspergillus* guideline recommends resistance testing in *A. fumigatus* and local resistance surveillance. If resistance rates exceed 10% liposomal amphotericin B or triazole and echinocandin first-line therapy should be considered.
Implications: Triazole resistance significantly complicates the management of aspergillosis and multidisciplinary research from a 'One-health' perspective is required to retain the triazole class for medical use. **P.P.A. Lestrade, Clin Microbiol Infect 2019;25:799**

Introduction

Aspergillus species are found worldwide, and billions of airborne spores are produced of which hundreds are inhaled daily by humans. Spores may reach the alveoli but are cleared by the immune system in healthy individuals. However, immunocompromised patients and those with structural lung disease are prone to develop *Aspergillus* infections, ranging from chronic pulmonary aspergillosis (CPA) to acute invasive aspergillosis (IA) [1]. In most regions of the world *Aspergillus fumigatus* is the most frequently encountered *Aspergillus* species, whereas in tropical climates *Aspergillus flavus* is the most common [2]. *Aspergillus terreus* has been reported at high frequency in specific centres, but is an infrequent cause of infection in most geographic regions [3].

Mortality in IA ranges from 20% to 30% with first-line voriconazole or isavuconazole therapy [4,5]. Although intrinsic antifungal drug resistance is recognized in certain *Aspergillus* species, acquired triazole resistance is an increasing concern, especially in *A. fumigatus* [6]. In this review we provide an update on recent insights in triazole resistance in aspergillosis and the challenges for patient management.

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Development of azole resistance

Changes in the taxonomic classification of *Aspergillus* species, with the recognition of sibling species, have complicated laboratory identification [7]. *Aspergillus fumigatus sensu stricto* is intrinsically susceptible to triazoles, but other species in this complex, e.g. *Aspergillus lentulus*, may be intrinsically resistant to triazoles and amphotericin B. Species outside the *A. fumigatus* species complex can also exhibit higher intrinsic resistance levels, e.g. some species in the *Aspergillus niger* complex, but are less often involved in IA [8].

Triazoles are not known to be mutagenic and therefore resistance is selected when genetic variation occurs in the progeny of *Aspergillus* species. In *A. fumigatus*, three modes of reproduction are recognized, including asexual, sexual and parasexual reproduction, of which the asexual cycle is abundant in nature [9]. Although the progeny of asexual reproduction is clonal, spontaneous mutations may occur. By chance, one of the mutated spores might be better suited to withstand specific stress factors such as triazole exposure. If the mutant spore develops in a triazole-containing environment, the isolate will have an advantage over wild-type spores and proliferate. The process of selection of resistant strains can take place in any triazole-containing environment. In *A. fumigatus*, generally, two resistance-selection environments are recognized: triazole-treated patients and environments that contain triazole fungicide residues [6]. Resistance selection may occur in patients with pulmonary cavities in which *A. fumigatus* can undergo asexual sporulation, and selection takes place through triazole therapy. Resistance mutations are commonly observed in the *Cyp51A* gene, which is the target of antifungal triazoles, and are characterized by single nucleotide changes, e.g. G54, M220, G138 [10,11]. In some patients, multiple resistance mutations may be present in clinical samples [10,11]. In culture, resistant isolates may exhibit a fitness cost, such as poor growth or lack of sporulation [12]. Single nucleotide resistance mutations typically occur in patients with chronic lung disease, such as chronic obstructive pulmonary disease and CPA, who receive long-term triazole therapy [11]. Triazole resistance was found to develop in 13% of individuals with CPA treated with itraconazole and in 5% for voriconazole [13], indicating that resistance selection is not infrequent.

The other resistance-selection environment is organic material that contains triazole fungicide residues [14,15]. *Aspergillus fumigatus* is not a phytopathogen and so is not the target for fungicide application, but many triazole fungicides where found to exhibit activity against *A. fumigatus* [16,17]. The fungus reproduces abundantly in organic material and recent studies indicate that resistance is selected if triazole fungicide residues are present in soil [18,19]. Resistant spores are released into the ambient air and subsequently inhaled by humans. In immunocompromised patients, triazole-resistant *Aspergillus* disease may develop, even in those that have not previously been exposed to triazoles [20]. In these cases, triazole resistance is often caused by specific amino acid changes in the Cyp51A protein in combination with tandem repeats (TR) in the gene promoter, e.g. TR_{34}/L98H and TR_{46}/Y121F/T289A [21,22]. Unlike patient-derived resistant isolates, environmental resistant isolates appear to have overcome any fitness cost, as they evolved in competition with wild-type isolates and so acquired similar fitness levels [12]. As patients will inhale both triazole-susceptible and triazole-resistant spores, mixed infections have been reported in patients with pulmonary IA [23,24].

In addition to *Cyp51A*-mediated resistance, various other resistance mechanisms have been described, including mutations in other genes and efflux pumps [25].

Resistance phenotypes

Resistance is frequently caused by mutations in the *Cyp51A* gene, encoding an enzyme involved in cell membrane synthesis [26,27], and the resistance phenotype depends on the underlying mutations. G54 mutations, for instance, cause resistance to itraconazole and posaconazole, but do not affect the activity of voriconazole and isavuconazole [26,28]. Furthermore, resistance to voriconazole is associated with resistance to isavuconazole [29]. In isolates harbouring a TR, over-expression of the *Cyp51A* gene was shown to contribute to the resistance phenotype [18,21]. TR_{34}-isolates show high resistance to itraconazole: MIC $\geq$16 mg/L, whereas TR_{46}-isolates are highly resistant to voriconazole [30]. However, most TR-isolates exhibit a pan-azole-resistant phenotype [30]. Of the four triazoles with anti-*Aspergillus* activity, posaconazole retains the lowest MICs in resistant isolates, usually one or two dilution steps above the resistance breakpoint of 0.25 mg/L [30].

Although triazole resistance has become a major concern in *A. fumigatus*, acquired resistance has been documented in other *Aspergillus* species. In *A. flavus*, point mutations in *Cyp51A*, *Cyp51B* or *Cyp51C* have been associated with triazole resistance [31]. In other isolates, up-regulation of *Cyp51* was observed, together with non-*Cyp51*-mediated mechanisms, e.g. efflux [31]. In *A. terreus*, triazole resistance seems to be limited to posaconazole and is attributed to mutations in *Cyp51A*, e.g. M217 [32]; however, in many posaconazole-resistant isolates no mutations were found [33].

Diagnosis of resistance

Species identification is an important first step in resistance detection in *Aspergillus* species, as this will help to identify intrinsically resistant species. The ESCMID-ECMM-ERS *Aspergillus* guideline recommends species identification to the complex level for clinically relevant isolates from patients requiring antifungal treatment [34]. Although growth at 48°C is indicative of *A. fumigatus sensu stricto*, selected isolates could be sent to mycology reference laboratories for identification beyond the species complex level.

Reference methods for MIC testing of moulds are available, i.e. the European Committee on Antimicrobial Susceptibility Testing and the Clinical and Laboratory Standards Institute, but many clinical microbiology laboratories do not routinely perform *in vitro* susceptibility testing of moulds [35]. Antifungal susceptibility testing of *Aspergillus* isolates is recommended in individuals with IA with the exception of triazole-naive patients in regions without resistance found in surveillance programmes [34]. The Infectious Diseases Society of America does not recommend routine MIC testing, except for patients that fail on antifungal therapy and in those that are thought to have a resistant infection [36]. Both guidelines recommend resistance testing for epidemiological purposes.

Clinical breakpoints and epidemiological cut-offs have been established for many drug–fungus combinations but they rely on reference methods, which are not widely available. Another problem is that different resistance phenotypes might be present in a single culture, necessitating testing of multiple (up to five) *A. fumigatus* colonies from a single sample [37]. To avoid the burden of multiple MIC-tests a screening agar was developed and validated that allows screening of multiple colonies for resistance (VIPcheck™, MediaProducts, Groningen, the Netherlands) [38,39]. The screening agar showed excellent sensitivity and specificity and so helps to select colonies that require MIC testing [38]. As *Aspergillus* culture is often negative in patients with IA, molecular tests have

been developed that enable the detection of one (TR_{34}/L98H; MycoGenie, Ademtech, France) or two (TR_{34}/L98H and TR_{46}/Y121F/T289A; AsperGenius, Pathofinder, the Netherlands; and Bruker Fungiplex Aspergillus Azole-R IVD PCR, Bruker, Germany) resistance mutations directly in clinical samples [40]. The performance of these assays was recently reviewed [40], showing advantages and disadvantages. The main advantage is the ability to detect resistance mutations in culture-negative patients and the AsperGenius assay also enables the detection of mixed infection [41]. An important drawback is the low sensitivity of the assays, because the *Cyp51A* gene is a single copy gene, and amplification of resistance markers may not be successful in 30% of bronchoalveolar lavage (BAL) samples [40,41]. Furthermore, only two resistance mutations are detected, which limits the use of the assay in some patient groups, such as those with CPA. A recent study showed that through pyrosequencing a broad range of resistance mutations could be detected directly in sputum from individuals with CPA [42], which might be a feasible strategy that needs further exploration.

Generally, resistance mutations correspond with a specific triazole phenotype. However, in patients who are chronically treated with triazoles, isolates may emerge with aberrant triazole phenotypes. This is probably caused by the accumulation of resistance mechanisms due to continued triazole exposure or switching between triazole compounds [12]. As many of these resistance mechanisms are unknown, resistance might be missed in culture-negative, PCR-negative patients, or if a resistance mutation is detected, the corresponding phenotype may be unpredictable.

Epidemiology

Resistance in *A. fumigatus* has spread globally, but the resistance frequencies vary considerably between geographic regions and between hospitals. An overview of countries reporting resistance in the environment and in clinical cultures is shown in Fig. 1 [17,24,25,43–110], Overall, resistance is dominated by environmental resistance mutations, especially TR_{34}/L98H [43]. A Dutch survey of unselected clinical *A. fumigatus* isolates showed that resistance increased from 7% (59/814 culture-positive patients; 95% CI 5.65–9.25) in 2014 to 15% (114/774 patients; 95% CI 10.71–15.42; p 0.001) in 2017 [109]. This basic surveillance network involves five university hospitals and screening of all *A. fumigatus* culture-positive clinical samples, but did not register underlying disease nor classify *Aspergillus* disease. In specific risk-groups in the Netherlands, such as intensive care unit patients, resistance frequencies exceeding 25% were found [35]. These frequencies, however, should be interpreted with caution because they include only *A. fumigatus* culture-positive patients. As most patients are culture-negative and diagnosed through detection of galactomannan, it remains unclear which proportion of culture-negative patients have resistant disease. There is significant variation in reported resistance frequencies, which might reflect true geographical variation, but may be due to other factors such as the denominator, resistance detection methods, or definition of triazole resistance [111,112]. A unified approach of reporting is warranted, as it will allow comparison of resistance frequencies between countries.

In addition, insight into the local epidemiology will help to guide local treatment recommendations, as experts have recommended to consider moving away from triazole monotherapy when the resistance frequency exceeds 10% [113]. A concern is that an accurate estimate of resistance frequency is not possible if a limited number of isolates are tested. The ESCMID-ECMM-ERS guideline recommends to test ≥100 *A. fumigatus* isolates [34], which is challenging for hospitals because it might take a long time to collect such a number of clinical isolates. Also, resistance frequencies may vary over time, further complicating an accurate estimate [114].

Clinical significance of resistance

Several case series showed mortality rates of 50%–100% in patients with triazole-resistant IA (Table 1) [11,20,43,115]. However, estimates of excess mortality can only be made when mortality

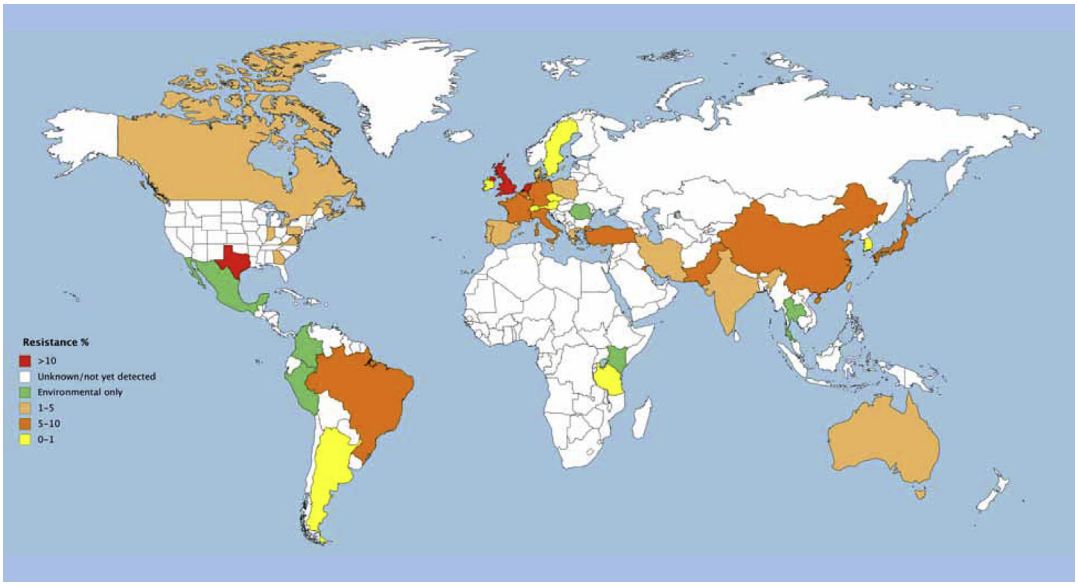

Fig. 1. Global epidemiology of azole resistance frequencies in clinical and environmental *A. fumigatus* isolates. Resistance prevalence was classified for clinical isolates. If only cases were reported they were classified as 0–1%. Average frequencies were calculated when multiple studies on resistance frequencies were available. The number of screened isolates varied as well as the number of studies that were performed in certain countries. Resistance rates may vary between regions within one country or vary for different patient groups. Many countries that have reported resistance in clinical isolates have also found triazole-resistant *A. fumigatus* in the environment, IE: Australia, Belgium, Brazil, China, Denmark, France, Germany, India, Iran, Ireland, Italy, Japan, Kuwait, the Netherlands, Switzerland, Taiwan, Tanzania, the United Kingdom and the United States of America. For references see text. Peru and Mexico were added based on personal communication with Agustin Reséndiz Sharpe.

Table 1
Clinical studies that report mortality in azole-resistant IA

Endpoint	Population	Study design	Mortality	Study
Failure and mortality (time unspecified)	Mixed, including IA, CPA and ABPA. Patients treated with ITZ (13/14) or VCZ (1/14).	Case series (multicenter). Culture positive with ITZ R according to EUCAST. Confirmation with Cyp51A-sequencing. Resistance phenotypic specified for ITZ, VCZ and POS.	2/2 (100%) in IA 4/12 (33%) in CPA/ABPA patients	[11]
Mortality at 12 weeks	IA. All patients were on VCZ therapy, 3 patients had combination with Ecand or L-AmB	Case series (multicenter). Culture positive with ITZ R, detected by screening with ITZ agar and confirmation with MIC and Cyp51A sequencing. VZC MICs also specified (6/8 R).	7/8 (90%) TRZ R 5/6 (80%) in patients with VCZ R	[20]
Failure and mortality (time unspecified)	Mixed, both IA, CPA and ABPA. Treatment unspecified.	Case series (multicenter). Culture positive with ITZ R using VIPcheck™ and confirmation with MIC and Cyp51A-sequencing. Resistance phenotype unspecified (ITZ and/or VCZ and/or POS resistance).	7/10 (70%) in IA 7/18 (39%) in CPA/ABPA	[43]
Mortality at 100 days after first isolation of resistant *A. fumigatus*	IA according to EORTC/MSG in HSCT recipients. Treatment regimes differed widely.	Case series (multicenter). Culture positive with ITZ R. Etest screening, MIC-testing and Cyp51A sequencing. Resistance phenotype specified for ITZ, VCZ and POS.	7/8 (88%)	[115]
Mortality and treatment failure at 6 weeks	Hematological patients with IA according to EORTC/MSG	Retrospective cohort (multicenter). Comparison of patients with resistance mutations detected by AsperGenius PCR and without resistance mutations directly in BAL	8/45 (19%) without mutations 4/8 (50%) with R mutations (P=0.07)	[41]
Mortality at 6 and 12 weeks after start antifungal therapy	IA according to EORTC/MSG or AspICU definitions.	Retrospective cohort (multicenter). Comparison of culture positive patients with and without phenotypically detected VCZ R by VIPcheck™ screening and confirmation with MIC according to EUCAST and Cyp51A-sequencing	44/158 (28%) in VCZ S 18/37 (49%) in VCZ R (P=0.017)	[116]
Mortality 6 weeks 12 weeks and 1 year after inclusion	Mixed, both IA, CPA, AB and ABPA. For IA EORTC/MSG and AspICU criteria were used. 4/5 patients with ITZ R IA were treated with VCZ.	Prospective cohort (multicenter). Comparison of culture positive patients with and without phenotypically detected ITZ R. Resistance phenotype unspecified (ITZ and/or VCZ and/or POS R) although MIC distributions were given. *Aspergillus* sp. other than *A fumigatus* also included.	Only 4 patients with classifiable IA had R strains, thus power was low. 1/4 (25%) died within 6 weeks. Mortality in the susceptible (classifiable) group was 29/72 (40%). No resistance was found In CPA.	[44]
Mortality 90 days after ICU admission	ICU patients with IA according to EORTC/MSG, or AspICU criteria.	Retrospective cohort (single center). Comparison of culture positive patients with and without phenotypically detected ITZ R by VIPcheck™ screening, MIC-testing according to EUCAST, and Cyp51A-sequencing. Resistance phenotype specified for ITZ, VCZ and POS.	10/10 (100%) R 23/26 (88%) S	[117]
Mortality and attributable mortality 42 days after first culture	Patients with hematological disease with IA according to EORTC/MSG. treatment in R group unspecified	Retrospective case control (single center). Comparison of culture positive patients with and without phenotypically detected ITZ R. MIC-testing using CLSI and Cyp51A-sequencing. Resistance phenotype specified for ITZ, VCZ and POS. *Aspergillus* sp. other than *A fumigatus* also included.	7/19 (37%) R all cause 30/83 (36%) S all cause 7/19 (37%) R attributable 26/83 (31)% S attributable	[58]
Failure and mortality and attributable mortality at day 14, 30 and 84	Hematology patients with AML and ALL and IA classification according to EORTC/MSG.	Prospective cohort study (multi center). This study included patients with possible aspergillosis. Comparison of culture positive patients with and without phenotypically detected ITZ R. Resistance screening with ITZ and VCZ using Etest MIC-testing according to EUCAST, and Cyp51A sequencing.	Only 25 patients with classifiable IA had positive cultures. Of these 2 patients had ITZ R. One patient, with ITZ R and VCZ I, survived (switch POS to L-AmB). Another patient, with ITZ R and VCZ R, died (switch VCZ prophylaxis x to L-AmB)	[60]
Mortality at 180 days after hematological diagnosis.	Patients with AML or MDS given chemotherapy or allogeneic HSCT, with IA according to EORTC/MSG.	Retrospective cohort study (single center). This study included patients with possible aspergillosis. Comparison of culture positive patients with and without phenotypically detected ITZ R. Resistance screening with VIPcheck™, MIC-testing according to EUCAST, and Cyp51A sequencing.	12 patients with IA were culture positive and only 2 patients had elevated MICs. One patient, VCZ intermediary R, survived (treated with VCZ). The other patient, ITZ R and VCZ S, died (treated with L-AmB)	[118]

IA invasive aspergillosis, CPA chronic pulmonary aspergillosis, ABPA allergic bronchopulmonary aspergillosis, AB Aspergillus bronchitis, S susceptible, R resistant/resistance, I intermediate, VCZ voriconazole, ITZ itraconazole, POS posaconazole, L-AmB liposomal amphotericin B, ECH echinocandins, TRZ triazoles.

rates of resistant cases are compared with those of susceptible IA. One study showed a 31% higher day-42 mortality in haematology patients with triazole resistance mutations detected by PCR in BAL compared with those with no resistance mutations in BAL [41]. A recent retrospective cohort study in *A. fumigatus* culture-positive patients with various underlying diseases showed a 21% higher day-42 mortality in patients with voriconazole-resistant IA compared with voriconazole-susceptible cases [116]. Importantly, a 23% higher day-42 mortality was found in patients that were started on inappropriate antifungal therapy, i.e. voriconazole in voriconazole-resistant IA, compared with those receiving initial appropriate antifungal therapy, i.e. voriconazole in voriconazole-susceptible infection, despite switching to appropriate antifungal therapy when triazole resistance was detected [116]. These clinical studies are in agreement with preclinical evidence that triazole resistance leads to excess mortality in triazole-treated patients [37]. In contrast, some studies reported no significant impact of resistance, but these studies were limited by low population sizes (Table 1) [44,58,60,117,118].

Challenges for patient management

The emergence of resistance challenges our current management strategies in patients with *Aspergillus* diseases. In individuals with CPA on triazole therapy, resistance testing of positive cultures is important to guide antifungal therapy. Management challenges include detection of resistance in culture-negative patients and the broad spectrum of possible resistance mutations. As previously mentioned sequencing-based resistance detection strategies might overcome these challenges [40,42]. In patients with IA, culture-positivity rates vary according to patient group and management strategy. In haematology patients the recovery rate of *A. fumigatus* from BAL can be very low [118], whereas >80% of individuals with influenza-associated aspergillosis were culture-positive [119]. As resistance in IA is almost exclusively caused by environmental resistance, the main challenge is to initiate appropriate antifungal therapy in individuals at risk for triazole-resistant infection. There are, however, no specific risk factors, so a 10% threshold may be used to determine appropriate initial antifungal therapy [34,113]. In regions exceeding 10% resistance, primary therapy with liposomal amphotericin B or voriconazole plus an echinocandin may be considered [34]. Initial resistance coverage in all individuals with IA may prevent excess mortality due to inappropriate therapy in triazole-resistant cases, but clinical evidence supporting this strategy is currently lacking. Furthermore, it remains unclear when de-escalation is safe, especially in individuals where triazole resistance has not been detected or ruled out. Optimal treatment of individuals with mixed triazole-resistant and triazole-susceptible infection remains undefined as well as the implications of triazole-resistance for the use of triazole prophylaxis. Breakthrough triazole-resistant IA has been reported in individuals on posaconazole prophylaxis [120], but animal experiments indicate that higher posaconazole exposure might prevent triazole-resistant infection [121].

Equally challenging is the optimal management strategy in regions with resistance rates $\leq$10%. In such regions triazoles remain the first-line treatment recommendation, but preventing excess mortality due to delay of appropriate antifungal therapy in sporadic triazole-resistant cases will be very difficult. Early detection of resistance would require intensive and rapid resistance diagnostics in all patients, while many laboratories currently lack the facilities to routinely perform such tests and the effort and costs needed to diagnose resistance might not outweigh the benefits of early appropriate therapy in sporadic resistant cases. A retrospective cohort study indicated that despite intensive resistance screening of *A. fumigatus* cultures, escalation to appropriate antifungal therapy occurred after a median of 10 days, which was associated with excess mortality [116]. Therefore any resistance management strategy would have to allow very early resistance detection, for instance through direct resistance PCR testing.

Conclusion

Triazole resistance in *A. fumigatus* is an increasing worldwide problem that causes major challenges in the management of individuals with *Aspergillus* diseases, as resistant infection increases the probability of treatment failure and mortality. There is a need for (inter)national resistance surveillance programmes as well as reliable and rapid diagnostic tests. Clinical studies are required to provide evidence for antifungal therapy strategies in triazole-resistant aspergillosis, and new antifungal drugs are needed with novel targets, that are effective in triazole-resistant infection. Furthermore, programmes aimed at understanding triazole resistance selection in the environment are paramount to reduce the burden of airborne infections with resistant isolates. However, effective interventions can be implemented only if the problem of triazole resistance in *A. fumigatus* is approached from a 'One-Health' perspective [122] and fungal resistance research is prioritized.

Transparency declaration

JFM received grants from F2G, Pulmocide and Amplyx. He has been a consultant to Scynexis and received speaker's fees from Merck, United Medical, TEVA and Gilead Sciences. PEV received grants from Merck, Pfizer, Gilead Sciences and F2G. He has been a consultant to Basilea, Scynexis, Merck, F2G and Siemens and received speaker's fees from Merck, Gilead Sciences and Pfizer.

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