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Review Article

Review Article

Triazole resistance surveillance in *Aspergillus fumigatus*

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Abstract

Triazole resistance is an increasing concern in the opportunistic mold *Aspergillus fumigatus*. Resistance can develop through exposure to azole compounds during azole therapy or in the environment. Resistance mutations are commonly found in the Cyp51A-gene, although other known and unknown resistance mechanisms may be present. Surveillance studies show triazole resistance in six continents, although the presence of resistance remains unknown in many countries. In most countries, resistance mutations associated with the environment dominate, but it remains unclear if these resistance traits predominately migrate or arise locally. Patients with triazole-resistant aspergillus disease may fail to antifungal therapy, but only a limited number of cohort studies have been performed that show conflicting results. Treatment failure might be due to diagnostic delay or due to the limited number of alternative treatment options. The ISHAM/ECMM *Aspergillus* Resistance Surveillance working group was set up to facilitate surveillance studies and stimulate international collaborations. Important aims are to determine the resistance epidemiology in countries where this information is currently lacking, to gain more insight in the clinical implications of triazole resistance through a registry and to unify nomenclature through consensus definitions.

Key words: *Aspergillus fumigatus*, aspergillosis, triazoles, resistance surveillance.

Introduction

Aspergillus fumigatus is a ubiquitous fungus that plays an important role in the degradation and recycling of organic matter. It can adapt to diverse ecosystems and conditions; in addition, it produces billions of spores that assure its survival and spread. Normally, it is not a primary human pathogen; however, with the increase of immunosuppressive therapies it has become an important cause of opportunistic infections.[1] The clinical spectrum of these fungi is diverse, ranging from allergic and chronic infections to acute invasive aspergillosis (IA).[2] High prevalence rates have been reported in specific patient populations like hematopoietic stem cell recipients (43%), solid organ transplant patients (19%), chronic pulmonary infections, and recently, in association with severe influenza pneumonia.[3–12] Currently, triazole antifungals are recommended as first choice for prophylaxis and treatment of aspergillus diseases.[13] However, since the first case of triazole-resistance in 1997, many centers around the world have reported resistance, threatening the current treatment against this fungus.[14–17]

Mechanisms of resistance selection

Triazole-resistance in *A. fumigatus* is defined as *in vitro* resistance of this fungus to at least one triazole antifungal agent or in vitro MIC values for this fungus that are higher than the epidemiological cutoff values of at least one triazole antifungal agent.[18] According to their phenotypic profiles these isolates can be grouped having resistance to a single triazole (e.g., voriconazole-resistant), to more than one azole (multi-triazole resistant) or to all clinically available azoles (pan-triazole resistant).[19] Triazole-resistance can be either intrinsic or acquired.

Intrinsic resistance is defined as the inherent resistance of all or almost all isolates of a single species to a certain drug without previous exposure to it.[20,21] Intrinsic resistance has been reported in cryptic species of the *Fumigati* species complex including *A. lentulus* or *A. calidoustus,* but not in *A. fumigatus* sensu strictu.[21] Azole compounds are not mutagenic, but resistance emerges through spontaneous mutations or recombination and subsequent selection following exposure to an antifungal drug (acquired resistance). Through asexual sporulation *A. fumigatus* produces abundant numbers of spores, many of which harbor spontaneous mutations, which ensures genetic diversity, and long-term azole exposure then selects offspring with the greatest ability to grow and reproduce in the presence of the azole.[16,22] Mechanisms of azole-resistance can be divided into 2 major groups: Cyp51A mediated and non-Cyp51A mediated.[15,23]

Triazole antifungals inhibit the biosynthesis of ergosterol, a component of the fungal cell membrane. They bind to the enzyme 14-alfa-demethylase (Cyp51) to interrupt the conversion of lanosterol to ergosterol.[24] The Cyp51 gene produces two isoforms of the enzyme, A and B. Cyp51A mutations may be either single-nucleotide polymorphisms (SNPs), tandem repeats in the gene promoter, or both. They usually affect affinity and not function, by modifying the binding site and restricting the entry of azoles.[25,26] The most common SNPs are G54, M220, G138, and G448, associated with different azole-resistant phenotypes.[16,27,28] Up to now, five tandem repeat mutations have been described. They cause overexpression of the Cyp51A gene products by promoter duplications. TR_{34}/L98H tandem repeat consists of an insertion into the promoter of two 34 base pair (bp) stretches in association with an amino acid substitution at codon 98.[29] This increases the protein level of expression and alters the docking of azoles, conferring pan-azole resistance with particularly high itraconazole MIC values.[30] The TR_{46}/Y121F/T289A mechanism consists of a 46 bp insertion in the promoter region and change of amino acids at codon 121 and 298,[31] conferring high-level voriconazole resistance and variable itraconazole MIC values.[32] Sometimes additional SNPs are found in the Cyp51A-gene, such as TR_{46}/Y121F/M172I/T289A/G448S. The third resistance mechanism involves a 53 bp tandem repeat insertion (TR_{53}) without mutations in the cyp51A gene, which confers a pan-azole-resistant phenotype.[33] Recently, two new mutations were found in the Netherlands consisting of three copies of the 46 bp tandem repeat ($TR_{46}{}^{3}$) and four copies ($TR_{46}{}^{4}$).[34]

Non-Cyp51A mutations are less characterized, and can be divided into four groups: efflux pumps, Cyp51B overexpression, cholesterol import, and HapE mutation. The efflux pumps are ATP binding cassette transporters whose function is to overcome intracellular toxin accumulation. Overexpression of two of these transporters is associated with azole-resistance, AfuMdr4 to voriconazole and Cdr113 to itraconazole.[35,36] Cyp51B-mediated azole resistance is rare; in two clinical isolates increased induction after exposure to itraconazole conferred resistance to azoles.[37] Regarding cholesterol import resistance, overexpression of Srba, a sterol regulatory element binding protein, increases resistance to fluconazole and voriconazole *in vitro*.[38,39] Lastly, after azole exposure, a P88L substitution in the CCAAT-binding transcription factor of the HapE gene conferred resistance against azoles. Only a few isolates have been reported from this mutation and *in vitro* studies suggest reduced fungal fitness as a consequence.[40] This list is not

exhaustive, and other isolates have as-yet unknown mechanisms of resistance.

Routes of azole resistance selection

As mentioned, azole-resistance is secondary to an acquired trait that occurs after azole exposure either after prolonged treatments (patient route) or after environmental exposure of *A. fumigatus* to fungicides (environmental route).

The patient route

Resistance develops after long term triazole treatment, and it is frequently seen in patients with aspergilloma, allergic and chronic aspergillosis, and predisposing conditions like lung cavities or cystic fibrosis.[15,16,22,41] Typically, the involved mechanisms are point mutations of the Cyp51A gene, often with various mutations developing within the same patient.[42,43] The in-patient resistance development and the capacity to acquire multiple resistance mechanisms were confirmed by genetic typing.[41] In patients with cavitary lesions asexual reproduction probably plays an important role in these mechanisms by facilitating the genetic transfer of the resistant mutation and the propagation of resistant spores.[15,44]

The environmental route

The isolation of azole-resistant strains from triazole-naive patients infers the possibility that patients could also acquire azole-resistant *A. fumigatus* from the environment. This is supported by several observations. First, the dominance of a few resistant mutations (mainly TR_{34}/L98H and TR_{46}/Y121F/T289A) found in more than 80% of clinical isolates from epidemiologically unrelated patients from different centers; where environmental and airborne isolates showed genetic clustering with these clinical isolates.[45–47] Second, geographical spread of these mutations to multiple countries and continents.[32,48,49] Third, presence of two or more genomic changes suggests a more complex reproductive method, like sexual reproduction, which has not been reported in human infection but is most likely to occur in the environment.[47] Finally, selection for azole-resistant *A. fumigatus* might occur after exposure to fungicides used routinely for crop protection and preservation of materials. Coincidentally, the first resistance report came shortly after the introduction of sterol-biosynthesis inhibiting (SBI) fungicides of the triazole class, which are molecularly similar to medical triazoles and to whom cross-resistance with medical triazoles has been documented.[47,50]

It remains unclear which applications of SBI-fungicides poses the greatest risk for accumulation of resistance mutations and subsequent infection of patients at-risk and which transmission routes are involved. In one study, the direct home environment of a patient with probable IA due to TR_{46}/Y121F/T289A was found to harbor isolates with the same resistance mutation and the same microsatellite genotype, providing further support for the environment as source for human infection.[51] Furthermore, plant bulbs containing TR_{46}/Y121F/T289A originating from the Netherlands were found to contaminate their direct environment once planted in the proximity of a hospital in Ireland.[52] However, the resistant isolates recovered from the environment were genetically different from those cultured from patients, indicating that other routes of transmission might contribute to human infection. Identifying sites with high burden of triazole-resistant *A. fumigatus*, exploring transmission routes of resistant spores and linking environmental source to clinical infection are areas where we need to increase our understanding. Nevertheless, the continued emergence of triazole resistance mutations in the environment suggests that our current practices of application of SBI-fungicides for crop protection and material preservation are generally non-durable. It is important to understand the pathophysiology of triazole resistance selection of *A. fumigatus* in the environment, as this will provide insights that can be used to implement preventive strategies. Such strategies might help to preserve the azole class for both human and animal disease management as well as agricultural applications.

Epidemiology

The first report of itraconazole-resistance came from two clinical isolates from California in 1997,[14] followed by sporadic reports from Sweden, Spain, Belgium, and France.[45] However, it was not until 2007 when a prospective study from the Netherlands described several triazole-resistant cases of *A. fumigatus* from patients with IA. Remarkably over 90% of these isolates had a predominant mutation, TR_{34}/L98H.[15] Since then, reports of triazole-resistant *A. fumigatus* have increased around the world.[53] The resistance frequencies vary according to the underlying condition of the patients, geographic region, the denominator used and laboratory techniques.[54] According to a multicenter international surveillance network, the incidence of triazole-resistance ranged between 0.6% and 4.2%, with TR_{34}/L98H mutation been the most frequent.[55] Reported resistance frequencies per country were: The Netherlands 0.8%–9.4% (but voriconazole resistance rates as high as 26% to 29% were reported from ICU patients from a single hospital), Belgium 5.5%, UK 6.6–27.8%, Germany 3.2% (but a high rate of 30% from hematopoietic stem cell

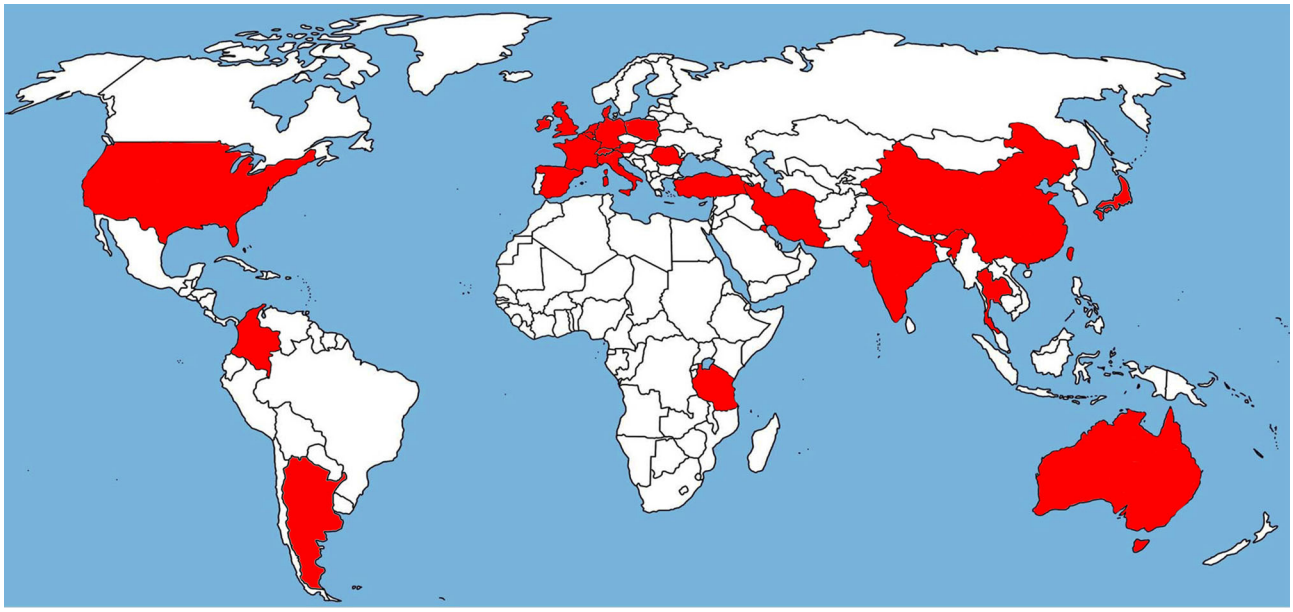

Figure 1. Countries that have reported triazole resistance in *A. fumigatus*. Countries with triazole resistance are depicted in red, while those with unknown resistance epidemiology are indicated in white.

transplant patients), Spain 0.3–4.2%, Denmark 4–6%, Greece 2.7%, Poland 4.13%, and Turkey 10.2%.[43,45,56–65]

Prevalence comparison between countries should be interpreted with caution as isolates origin, number of patients isolates included (single or multiple), and patients underlying conditions may differ among studies.

The first cases of TR_{34}/L98H mutation outside Europe were reported by the ARTEMIS Global Surveillance Study group in 2011 from Chinese isolates, with a prevalence of 5.8%.[66] Other countries that have reported resistance include India 1.7%, Iran 3.2%, Japan 6.1%, Thailand 3.2%, Australia 2.6%, Tanzania 13.9% (environmental), Colombia 9.3% (environmental), and the United States 0.6–11.8%.[67–70] However, MIC-testing of *A. fumigatus* is not routinely performed in many centers and therefore resistance is under diagnosed. Through testing of fungal culture collections it became apparent that the first TR_{34}/L98H isolate in Italy was cultured as early as 1998[71] and in the United States a TR_{46}/Y121F/T289A isolate was cultured already in 2008, 1 year before it was discovered in the Netherlands.[72] It remains unclear when and how triazole resistance emerged in *A. fumigatus*, and if resistance mutations developed once and subsequently migrated or that the same mutation developed on multiple occasions in different geographic locations. Genetic characterization and comparisons of resistant isolates from various parts of the world will help to gain further insights in the origin and migration of these traits. Although triazole resistance has now been found on six continents, the presence and frequency of resistance still remains unknown in many countries (Fig. 1).

Epidemiological data on triazole-resistance derives mainly from two settings: chronic aspergillus diseases (aspergilloma, chronic pulmonary aspergillosis, and chronic colonization) and acute IA. Resistance in patients with chronic aspergillus diseases can be secondary to point mutations of the *CYP51A* gene or from infection with an environmental triazole-resistant isolate. In patients with IA, resistance typically originates from isolates that are already triazole-resistant and from an environmental origin.[17,45] No discernible patient risk factors to predict resistant IA are known, as surveillance studies showed that up to two-thirds of patients with triazole-resistant IA had not been previously treated with a triazole.[46]

Clinical implications

Triazoles are currently the cornerstone for prophylaxis and treatment of aspergillus diseases. Itraconazole and posaconazole are used for chronic conditions and prophylaxis, whereas voriconazole and isavuconazole are the first line treatment against IA. Compared to other antifungal agents, triazole therapy is associated with better clinical response, improved survival, less infusion-related toxicity, and less nephrotoxicity.[73,74] Mortality from IA in bone marrow transplant patients before the use of voriconazole exceeded 70%. Since the introduction of voriconazole

Table 1. Interpretation of *A. fumigatus* MIC values for reference broth microdilution methods.

Strain	Antifungal	CLSI[2]	EUCAST[1] Clinical Breakpoint	
		ECOFFs	S ≤	R >
A. fumigatus				
	Itraconazole	1	1	2
	Posaconazole	0.25[2,3]	0.125	0.25
	Voriconazole	1	1	2
	Isavuconazole	1	1	1

1. EUCAST Antifungal Clinical Breakpoint Table v. 8.1 valid from 2017-03-01 (European Committee on Antimicrobial Susceptibility Testing).
2. CLSI Epidemiological Cutoff Values for antifungal susceptibility testing M38-2A, 2009 (Clinical Laboratory Standards Institute).
3. Pfaller MA, et al; Clinical and Laboratory Standards Institute Antifungal Testing Subcommittee. *J Clin Microbiol*. 2009 Oct; 47: 3142–3146. Rodriguez-Tudela JL, et al; *Antimicrob Agents Chemother*. 2008; 52: 468. Epub 2008.
ECOFF, epidemiological cutoff value; R, resistant; S, susceptible.
To simplify clinical breakpoints, EUCAST do not list the intermediate category. It is readily interpreted as the values between the S and the R breakpoints.

6-week and 12-week mortality rates have decreased to 21.5% and 35.5%, respectively.[75]

Unfortunately, treatment and overall patient's health is at risk by the emergence of resistance of *A. fumigatus*. The Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) defined epidemiological cutoff values and clinical breakpoints (based on MIC distribution, PK/PD of antifungal azoles, *in vivo* experimental correlation between SNPs and failure, and clinical experience) respectively for the interpretation of triazole resistance testing in *A. fumigatus* (Table 1).

Despite this, little is known about the implications of triazole-resistant aspergillus determination in clinical settings due to lack of prospective controlled clinical trials. Nonetheless, decreased survival has been observed when infection is induced by isolates with elevated MICs for itraconazole, posaconazole, voriconazole and isavuconazole in animal models.[76–80] Fitness and virulence of isolates that harbor TR_{34}/L98H mutations have no apparent cost compared to wild type strains, which might facilitate its survival and spread in the environment.[77] Mortality rates in case series of patients with triazole-resistant IA of hematology-oncology patients in the Netherlands and Germany was reported to be around 88%, but as many factors determine the outcome, including the underlying condition of the patient, the timing of antifungal therapy and drug exposure, it remained unclear if and to which extent triazole-resistance contributes to treatment failure.[46,81,82] To date six studies have compared the mortality of triazole-susceptible IA with that of triazole-resistant IA in cohorts of high-risk patients, of which two have only been presented as abstract (Table 2). The studies investigated different risk groups, used different diagnostic criteria, different endpoints for outcome and different treatment regimens, thus precluding direct comparisons between the studies. Two studies found a more than two-fold increased mortality rate in patients with triazole-resistant IA compared with susceptible disease.[83,84] However, the four other studies failed to find a difference between susceptible and resistant IA.[61,81,83,84] In two studies a high mortality rate was observed in patients with triazole-susceptible IA, which might be due to other variables such as the underlying condition or proportion of critically ill patients. Patients with IA directly admitted to the ICU were found to have a poorer outcome than those admitted to the hematology ward.[85] Also, the number of patients with documented triazole-resistant IA was low in each individual study, ranging between five and 19 patients in the reported studies (Table 2). Clearly, prospective multicenter trials or prospective registries are needed to further explore the clinical implications of triazole-resistance. With respect to chronic aspergillosis, case series also indicated an association between in vitro resistance and triazole treatment failure.[17,42,46]

Most isolates that harbor a resistance mutation exhibit a pan-triazole resistant phenotype, thereby virtually losing the triazole class as a whole for treatment.[42] The limited alternative treatment options might contribute to the high mortality rate observed in case series. Moreover, the triazoles have the advantage to have an oral formulation that allows outpatient treatment; loss of this oral route will increase patient hospital stay and associated risk of nosocomial infections and healthcare costs.

In addition to treatment failure, difficulty in diagnosing triazole resistance probably contributes to the high mortality rate. MIC-testing of moulds is not widely available in clinical microbiology laboratories, and isolates are often sent to mycology reference laboratories, which causes delay. Furthermore, detection of resistance largely relies on culture, as *A. fumigatus* colonies can be subjected to MIC-testing. However, *Aspergillus* culture is positive at best in only 25% to 50% of patients, and many patients are diagnosed through detection of the biomarker galactomannan and computed tomography of the chest. Neither of these provide information on the *Aspergillus* species that is causing the disease, let alone the *in vitro* susceptibility. Furthermore, patients with mixed infection due to both triazole-susceptible and triazole-resistant isolates have been reported,[86] and both susceptible and resistant *A. fumigatus* colonies may be present in culture.[87] Therefore, triazole-resistant cases may be misdiagnosed unless

Table 2. Mortality in cohort studies comparing the outcomes of triazole-susceptible IA with that of triazole-resistant IA.

Study design	Patient population	Outcome parameter	Triazole susceptible group	Triazole resistant group	Significance	Comment	Reference
Multicenter retrospective	Hematology	Treatment failure	12/45 (26.7%)	6/8 (75%)	$P = .01$	PCR-based detection of resistance in *A. fumigatus*	(83)
		Mortality (day 42)	8/45 (18.6%)	4/8 (50%)	$P = .01$	PCR-based detection of resistance in *A. fumigatus*	
Single center retrospective	Hematology	Mortality (day42)	30/83 (36%)	7/19 (37%)	NS	Multiple *Aspergillus* sp.; culture-positive patients	(85)
Single center retrospective	ICU	Mortality (day 90)	23/26 (88%)	10/10 (100%)	NS	*A. fumigatus* culture-positive patients	(82)
Single center retrospective	All risk groups	Mortality (day 90)	21/58 (36.2%)	6/7 (85.7%)	$P = .0174$	*A. fumigatus* culture-positive patients	(84)
Single center retrospective	All risk groups	Mortality (day 90)	25/43 (58.1%)	10/19 (52.6%)	NS	*A. fumigatus* culture-positive patients	(84)
Multicenter prospective	All risk groups	Mortality (day 42)	58/122 (47.5%)	2/5 (40%)	NS	*A. fumigatus* culture-positive patients	(62)

ICU, intensive care unit; NS, not significant.

multiple colonies are analyzed.[88] Recently, polymerase chain reaction (PCR) tests have become available that enable the detection of *Aspergillus* species and resistance markers directly in clinical specimens such as BAL-fluid.[89] Initial validation studies indicate that triazole resistance mutations can be detected in culture-negative patients, thus increasing our diagnostic yield.[83]

Triazole resistance in *A. fumigatus* has emerged on a global scale with the environmental route of resistance selection as major driver for geographical migration and clinical disease. Triazole-resistant *Aspergillus* disease is difficult to manage due to diagnostic challenges and the limited arsenal of alternative antifungal drugs with anti-*Aspergillus* efficacy. Although in the past decade many researchers have investigated this problem from different angles, ranging from resistance genetics to patient management, many questions remain unresolved.

Given the many unanswered questions regarding the epidemiology, pathophysiology, risk factors, diagnostic strategies, and treatment options for triazole resistance in *A. fumigatus,* a working group was initiated by the International Society for Human and Animal Mycology (ISHAM) and the European Confederation for Medical Mycology (ECMM), the ISHAM/ECMM *Aspergillus* Resistance Surveillance working group. Within this working group research questions were discussed and prioritized that need to be addressed in the near future. The meeting was held in January 2017 and was attended by 43 group members from 15 countries.

One important aim is to gain more insight into the epidemiology of triazole resistance in *A. fumigatus*. Several reviews have presented a world map with the countries that have reported triazole resistance in red, similar to Figure 1. However, most areas of the world are white, indicating that there is no information published on triazole resistance for that country. The ISHAM/ECMM *Aspergillus* Resistance Surveillance working group therefore has started an epidemiological survey entitled "turn the world red." This study aims to collect resistance data for all countries that currently lack information on triazole resistance. The ISHAM/ECMM *Aspergillus* Resistance Surveillance working group aims to collaborate with the ESCMID Fungal Infection Study Group (EFISG) in order to maximally benefit from existing collaborations of the members or their institutes with different countries. Through these collaborations, we hope to be able to contact local or national mycology centers, if present, and assist in the collection and analysis of *A. fumigatus* isolates, originating either from the environment or from clinical samples. If possible, we aim to determine the underlying resistance mutations. Reports on antimicrobial resistance generally stress the importance of

resistance surveillance, and through this initiative we aim to significantly increase our knowledge as well as set up a network that can be used for continued surveillance in the future.

A second important aim is to study and improve our ability to detect triazole resistance in *A. fumigatus*. Through a questionnaire, data will be collected on the number of *A. fumigatus* isolates that have been cultured, the procedures that are followed for identification and in vitro susceptibility testing, and the frequency of triazole resistance and underlying resistance mutations. We aim to also collect information on the underlying diseases of patients with positive *Aspergillus* culture. This working group network can also be used to sample specific niches or collect and test certain products for the presence of triazole resistance or to send out proficiency panels.

A prospective registry will commence aimed at collecting clinical data from patients with triazole-susceptible and triazole-resistant invasive aspergillosis in hematology patients. This study will help to further explore the implications of triazole resistance in patients with hematological malignancy.

Another area of interest is to propose unified definitions regarding triazole resistance. A paper was published in 2009 that proposed a nomenclature for triazole resistance in *Aspergillus*, but given the increased insights in resistance genotypes, MIC distributions and advances made in clinical breakpoints, an update is warranted.[88] Several issues remain unclear in the literature and would benefit from unified nomenclature. For instance, the definitions and nomenclature of target site mutations, such as the number of tandem repeats or the distinction between polymorphism and resistance mutation.

Finally, there is a significant proportion of clinical and environmental *A. fumigatus* isolates with a triazole resistant phenotype but with a wild-type *CYP51A* gene. This suggests other yet unknown resistance mutations. Through collaboration and sharing of resistance phenotypes and of sequence information we aim to gain more insight in the underlying mutations in these isolates. Our ability to identify resistance hotspots other than the *CYP51A* gene would help to improve molecular based resistance detection formats.

Declaration of interest

A.R.S. has received a FLOF scholarship KU Leuven; K.L. has received research grants, travel support and lecture honoraria from Gilead, MSD and Pfizer; J.F.M. received grants from Astellas, Basilea, F2G and Merck. He has been a consultant to Astellas, Basilea, and Merck and received speaker's fees from Merck, Gilead and United Medical. P.E.V. has received research grants and served as consultant for Gilead Sciences, Pfizer, MSD, Astellas, F2G, and Basilea.

All other authors report no conflicts of interest.

The authors alone are responsible for the content and the writing of the paper.

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