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Prospective multicentre study on azole resistance in *Aspergillus* isolates from surveillance cultures in haematological patients in Italy

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ABSTRACT

Objectives: This study was conducted to assess the prevalence of azole resistance in *Aspergillus* isolates from patients with haematological malignancies or who were undergoing haematopoietic stem cell transplantation and to identify the molecular mechanism of resistance.
Methods: In this 28-month prospective study involving 18 Italian centres, *Aspergillus* isolates from surveillance cultures were collected and screened for azole resistance, and mutations in the *cyp51A* gene were identified. Resistant isolates were genotyped by microsatellite analysis, and the allelic profiles were compared with those of resistant environmental and clinical isolates from the same geographical area that had been previously genotyped.
Results: There were 292 *Aspergillus* isolates collected from 228 patients. The isolates belonged mainly to the section *Fumigati* (45.9%), *Nigri* (20.9%), *Flavi* (16.8%) and *Terrei* (4.8%). Three isolates showed itraconazole resistance: *Aspergillus fumigatus sensu stricto*, *Aspergillus lentulus* (section *Fumigati*) and *Aspergillus awamori* (section *Nigri*). The itraconazole resistance rates were 1% and 1.48% considering all *Aspergillus* spp. isolates and the *Aspergillus* section *Fumigati*, respectively. The prevalence of azole resistance among all the patients was 1.3%. Among patients harbouring *A. fumigatus sensu stricto* isolates, the resistance rate was 0.79%. The *A. fumigatus* isolate, with the TR_{34}/L98H mutation, was genotypically distant from the environmental and clinical strains previously genotyped.

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Conclusions: In this study, the *Aspergillus* azole resistance rate was 1% (3/292). In addition to *A. fumigatus sensu stricto*, *A. lentulus* and *A. awamori* azole-resistant isolates were identified. Therefore, it is important have a correct identification at the species level to address a rapid therapy better, quickly understand the shift towards cryptic species and have an updated knowledge of the local epidemiology.

1. Introduction

Several fungal infections in the compromised host arise from the inhalation of spores as a consequence of environmental exposure. Fungal colonization can be acquired in the community, and it might evolve into an invasive infection during hospitalization due to severe immunosuppressive treatments to which patients are exposed [1].

Invasive aspergillosis (IA) is a severe disease in patients with haematological malignancy and in those undergoing haematopoietic stem cell transplantation (HSCT) and azoles, mainly voriconazole and posaconazole, are the most widely used antifungals in prophylaxis and targeted therapy [2]. The difficulties of obtaining biological samples, such as via bronchoalveolar lavage or biopsy in severely immunocompromised patients who are often thrombocytopenic, limit the possibility of culturing the fungus. For this reason, the diagnosis of IA in haematological patients relies mainly on indirect methods (e.g. galactomannan assay and polymerase chain reaction) [2]. In several haematological and transplantation centres, surveillance cultures of nasal and oropharyngeal swabs are routinely carried out because the colonization of the upper airways represents the first step in the development of a pulmonary infection [3,4].

The emergence of multi-azole-resistance has been recognized worldwide with the presence of resistant *Aspergillus fumigatus* environmental isolates. These appeared as a consequence of the wide use of fungicides in agriculture and for the preservation of wood, leather or paper [5–10]. In Italy, recent studies indicate that azole-resistant isolates were cultured from 16.9% of soil samples [11,12]. In particular, azole resistance in *A. fumigatus* is mainly associated with several point mutations in the *cyp51A* gene. In the environment, the dominant mechanism of resistance, a TR_{34}/L98H mutation in the *cyp51A* gene, is the same as the most frequent one in azole-naïve patients, suggesting an environmental origin of resistance in clinical isolates [13–16]. Two recent Dutch studies have shown a variability in the frequency of azole resistance in different patient populations: 4.5–26% in isolates from intensive care unit (ICU) patients with a probable IA and 24.6% in isolates from haematological patients [17,18]. In Italy, a retrospective study conducted on a heterogeneous population of patients reported an azole resistance rate of 6.25% in the period 1998–2006 [19].

Azole resistance has important clinical consequences, reducing therapeutic options and limiting the probability of effective prophylaxis [13]. Patients with IA caused by a multiazole-resistant strain have a mortality rate of 88% compared with 30–50% in patients infected by a susceptible strain [7].

A 28-month, prospective multicentre study (ARTE study) was conducted in Italy to assess the prevalence of azole resistance in *Aspergillus* isolates from patients with haematological malignancies or who were undergoing HSCT. This study was carried out to identify the molecular mechanism of resistance, compare the genotypes of environmental and clinical isolates from the same geographical area and correlate resistance to demographic or behavioural variables and to antifungal treatments.

2. Methods

2.1. Isolates and patients

Aspergillus spp. clinical isolates (292) were collected in the period September 2014 to December 2016 in 18 Italian hospitals. The isolates were obtained through surveillance cultures in patients with haematological diseases and HSCT recipients, mainly from respiratory tract samples—nasal swab (124 isolates), sputum (76 isolates), pharyngeal swab (20 isolates) and auricular swabs ($n = 2$). Additionally, 24 isolates were obtained through diagnostic procedures, such as bronchoalveolar lavage ($n = 17$), tracheal aspirates ($n = 6$), and nasal biopsy ($n = 1$). For some samples, more than one isolate was analysed as a different species or as having different morphology.

Demographic characteristics, underlying disease, previous antifungal treatments, smoking habits, living in rural areas and work or recreational activity with soil exposure were collected in an anonymized form.

2.2. Identification and screening for azole resistance

The isolates, identified from the hospital microbiology laboratories as *Aspergillus* spp. on the basis of macroscopic and microscopic morphology, were sent to the reference laboratory (Medical Mycology Laboratory of Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy) for the screening of azole resistance.

Briefly, conidia from a 3-day-old culture on Sabouraud dextrose agar (SDA; Biolife, Milan, Italy) were suspended (ca. 0.5 McFarland standard) in sterile distilled water added to 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO). The suspension was inoculated onto three plates containing SDA supplemented with 4 mg/L itraconazole (Sigma-Aldrich), 1 mg/L voriconazole (Sigma-Aldrich) and 0.5 mg/L posaconazole (Sigma-Aldrich) and onto one plate of SDA used as a control [19]. The plates were examined after 48 h of incubation at 37 °C.

2.3. Susceptibility testing

Isolates able to grow on azole-containing agar plates were tested by the broth microdilution method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology [20]. The minimal inhibitory concentration (MIC) of itraconazole, voriconazole and posaconazole was determined visually as the lowest concentration of the drug yielding complete inhibition of fungal growth. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality controls. Isolates with an MIC of itraconazole and voriconazole $\geq$4 mg/L and those with an MIC of posaconazole $\geq$0.5 mg/L were considered resistant, whereas those with an MIC of itraconazole and voriconazole $\leq$1 mg/L and those with an MIC of posaconazole $\leq$0.125 mg/L were considered susceptible [21].

2.4. Genotyping testing and cyp51A sequencing

Genomic DNA was extracted from the *Aspergillus* spp., azole-resistant isolates using PrepMan® Ultra sample preparation

reagent (Applied Biosystems, Foster City, CA). *A. fumigatus* isolates were identified by amplification and sequencing of a portion of the β-tubulin gene [11]; for the molecular identification of *Aspergillus* section *Nigri*, primers Bt2a and Bt2b specific for the β-tubulin gene were used [22].

To identify mutations responsible for *A. fumigatus* azole resistance, the *cyp51A* gene promoter was amplified using primers PA5 and PA7 [23]. The *cyp51A* gene was amplified as described previously [11].

Amplicons of the *cyp51A* gene were sequenced using BigDye™ terminators (Applied Biosystems) in an ABI PRISM1 310 Genetic Analyzer (Applied Biosystems); nucleotide sequences were analysed using Finch TV software v.1.4.0 (Geospiza; https://digitalworldbiology.com/FinchTV). The sequence alignment of Cyp51AF1/R3, Cyp51AF2/R2 and consensus Cyp51AF1R3/AF2R2 were determined using EMBOSS explorer (http://www.bioinformatics.nl/emboss-explorer to obtain the entire sequence of the *cyp51A* gene fragment (1168 bp). The Cyp51AF1/R2 sequences, obtained from resistant strains, were aligned using the Clustal W algorithm (https://www.ebi.ac.uk/Tools/msa/clustalou) using the *cyp51A* gene sequence of *A. fumigatus* strain 237 (GenBank accession no. AF338659) as a wild-type reference.

2.5. Microsatellite analysis

Resistant isolates were genotyped by microsatellite analysis using the primers STRAf3A, STRAf3B, STRAf3C, STRAf4A, STRAf4B and STRAf4C, as previously described [24]. The allelic profiles were compared with those of resistant isolates previously genotyped [11]. To compare the results of clinical isolates and environmental isolates, the isolate IUM 11-0396 was used to normalize the sizes. Microsatellite profiles were imported into GenAlEx 6.5 [25] for Nei's genetic distance matrix calculation. MEGA software (version 4.0) [26] was used to design a dendrogram by the UPGMA (unweighted pair group method using arithmetic averages) clustering algorithm using the distance matrix generated by GenAlEx 6.5.

3. Results

During the 28-month, prospective, multicentre cohort study, a total of 292 *Aspergillus* spp. isolates from 228 haematological or

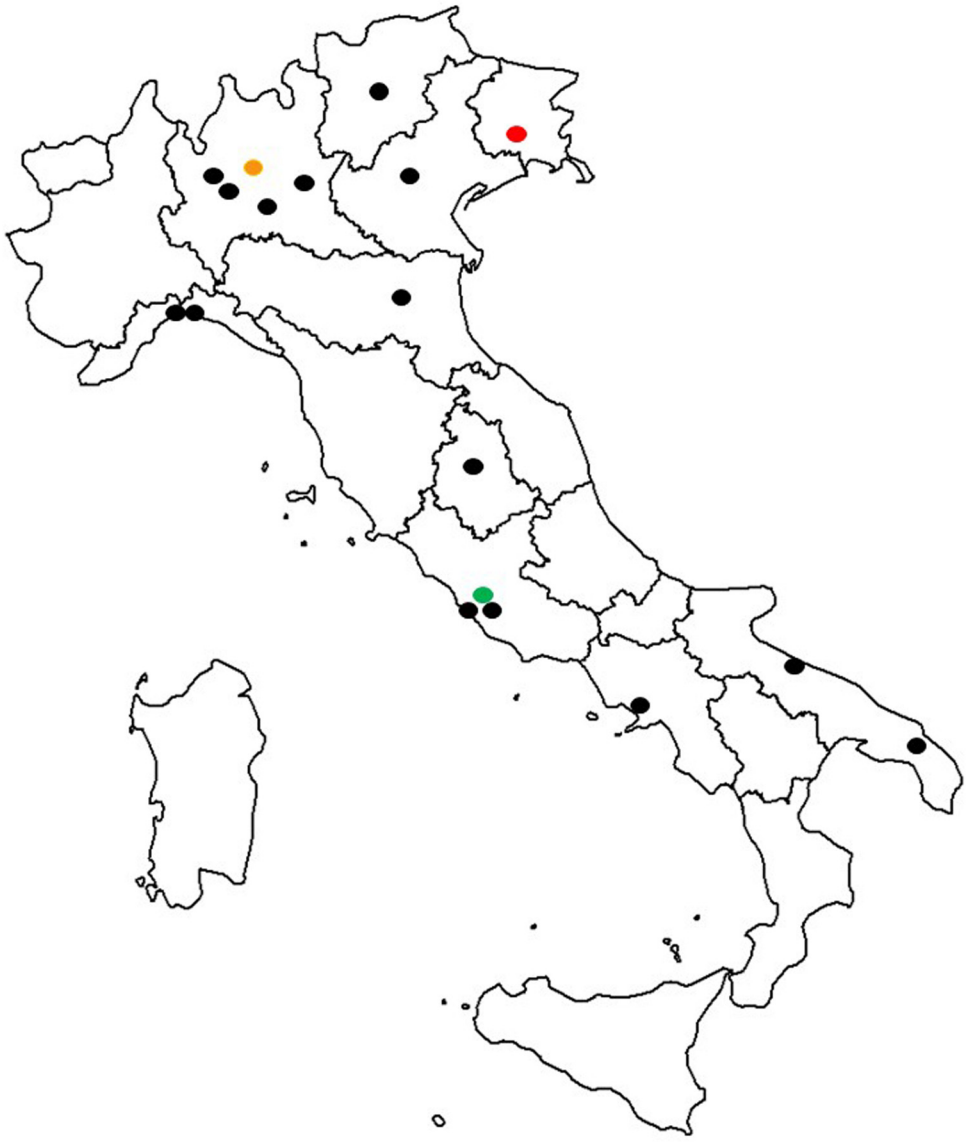

Fig. 1. Geographical distribution of involved hospitals. Coloured points indicate the sites at which the resistant strains were isolated: *Aspergillus awamori*, red dot; *A. fumigatus sensu stricto*, orange dot; *A. lentulus*, green dot.

Table 1
Distribution of *Aspergillus* spp. isolates from 228 haematological or HSCT patients.

Section	Species	Isolates	
		No.	**%**
Fumigati	*A. fumigatus*	134	45.9
	A. lentulus	1	0.3
Nigri	*A. niger*	59	20.2
	A. awamori	2	0.7
Flavi	*A. flavus*	50	17.1
	A. oryzae	6	2.0
Terrei	*A. terreus*	14	4.8
Nidulantes	*A. nidulans*	6	2.0
	A. unguis	1	0.3
Aspergillus	*A. glaucus*	4	1.4
	Eurotium amstelodami	2	0.7
Circumdati	*A. ochraceus*	4	1.4
Restricti	*A. restrictus*	1	0.3
	A. penicillioides	5	1.7
Versicolores	*A. versicolor*	3	0.1
Total		292	

HSCT, haematopoietic stem cell transplantation.

HSCT patients were collected in 18 hospitals located in northern (11 centres), central (4 centres) and southern (3 centres) Italy (Fig. 1).

The mean age of the patients was 55 years (range, 3–63 years), 60% were males, and the underlying diseases were acute lymphatic leukaemia (10.5%; $n = 24$), chronic lymphatic leukaemia (4.4%; $n = 10$), acute myeloid leukaemia (32%; $n = 73$), chronic myeloid leukaemia (0.9%; $n = 2$), hairy cell leukaemia (0.4%; $n = 1$), lymphoma (22.8%; $n = 52$), multiple myeloma (12.7%; $n = 29$) and other haematological malignancies (7.9%; $n = 18$); 40.8% (93/228) of patients underwent HSCT (67.7%, $n = 63$, allogeneic; 27.9%, $n = 26$, autologous). Prior to *Aspergillus* isolation, 40.4% (92/228) of patients received a previous azole treatment as prophylaxis or target therapy, 6 received treatment with liposomal amphotericin B or echinocandins and 28 did not receive any antifungal treatment before the fungal isolation. No information was available for the other patients.

Information about smoking habits and working and recreational activity with soil exposure was limited: 26 of 44 respondents were smokers and 22 handled soil. A total of 162 patients gave information about their place of residence—94 (58%) reported living in a rural area and 68 (42%) in an urban area.

The most frequently isolated species belonged to the section *Fumigati* (45.9%), *Nigri* (20.9%), *Flavi* (16.8%) and *Terrei* (4.8%). A detailed species distribution is reported in Table 1.

The screening for azole resistance revealed that 21 of 292 (7.2%) isolates were potentially resistant—14 *Aspergillus* section *Nigri*, 6 *Aspergillus* section *Fumigati* and 1 *Aspergillus* section *Flavi*. The broth microdilution method confirmed azole resistance in three isolates from three patients—one *A. fumigatus sensu stricto* (itraconazole MIC, 4 mg/L; posaconazole MIC, 0.5 mg/L), one *A. lentulus* (section *Fumigati,* itraconazole MIC > 16 mg/L) and one *Aspergillus awamori* (section *Nigri*, itraconazole MIC > 16 mg/L) (Table 2). In Fig. 1, the places where the resistant strains were isolated are reported.

The itraconazole resistance rate was 1% (3/292) considering all *Aspergillus* spp. isolates and 1.48% (2/135) considering only *Aspergillus* section *Fumigati*. The prevalence of azole resistance among all the patients was 1.3% (3/228). The prevalence was 0.79% (1/126), considering only patients harbouring *A. fumigatus sensu stricto* isolates.

The *A. fumigatus sensu stricto* resistant strain was isolated from one patient (male, 55 years old, lived in the countryside, smoker) with myeloma who underwent autologous HSCT (Table 2). The patient was diagnosed with probable pulmonary IA with a positive galactomannan antigen in bronchoalveolar lavage. The patient had received fluconazole prophylaxis and voriconazole as treatment following *Aspergillus* isolation in culture. Molecular analysis revealed a TR_{34}/L98H mutation in the *cyp51A* gene. Microsatellite analysis showed that this *A. fumigatus* clinical isolate was genotypically distant from the environmental and clinical isolates from other clinical strains from the same geographical area (Fig. 2).

Both *A. lentulus* and *A. awamori* isolates showed high MIC values of itraconazole (MIC > 16 mg/L); *A. lentulus* also showed high values of voriconazole (2 mg/L). More details regarding these patients are shown in Table 2. Note that the patient with *A. awamori* culture had gardening as a possible risk factor. Mutations responsible for azole resistance are not known for these species [27].

4. Discussion

A. fumigatus azole resistance, both in clinical and environmental isolates, is widely studied and is a source of worldwide concern because azole therapy can be compromised, especially in the most critical patients, such as patients with haematological malignancies. The main aim of this prospective multicentre cohort study was to establish the prevalence of *A. fumigatus* azole-resistant isolates in patients affected by haematological malignancies or who had undergone HSCT, hospitalized in different Italian centres.

Azole resistance was identified in three patients infected or colonized by an *Aspergillus* spp., resulting in a rate of 1.3%. The rate

Table 2
Azole-resistant *Aspergillus* isolates and patients demographic and clinical characteristics.

Parameter	Patient 1	Patient 2	Patient 3
Aspergillus spp.	*A. awamori* (section *Nigri*)	*A. fumigatus sensu stricto*	*A. lentulus*(section *Fumigati*)
Specimen	Nasal swab	Bronchoalveolar lavage	Sputum
Reason for culture	Diagnosis	Diagnosis	Surveillance
Aspergillosis	Sinusitis	Probable pulmonary	Colonization
Minimal inhibitory concentration (mg/L)			
ITRA	>16	4	>16
POSA	0.12	0.5	0.03
VORI	0.5	0.5	2
Cyp51A mutation	Not known	TR_{34}/L98H	Not known
Gender, age (years)	Male, 72	Male, 55	Female, 63
Underlying disease	Acute myeloid leukaemia	Myeloma; auto- HSCT	Severe aplastic anaemia
Possible predisposing factors	Gardening	Living in countryside; smoking habit	Nothing declared
Antifungal administration prior to *Aspergillus* isolation	Fluconazole	Fluconazole	None
Treatment after *Aspergillus* isolation	Voriconazole	Voriconazole	None
Outcome at 6 months after isolation	Alive	Alive	Died

HSCT, haematopoietic stem cell transplantation; ITRA, itraconazole; POSA, posaconazole; VORI, voriconazole.

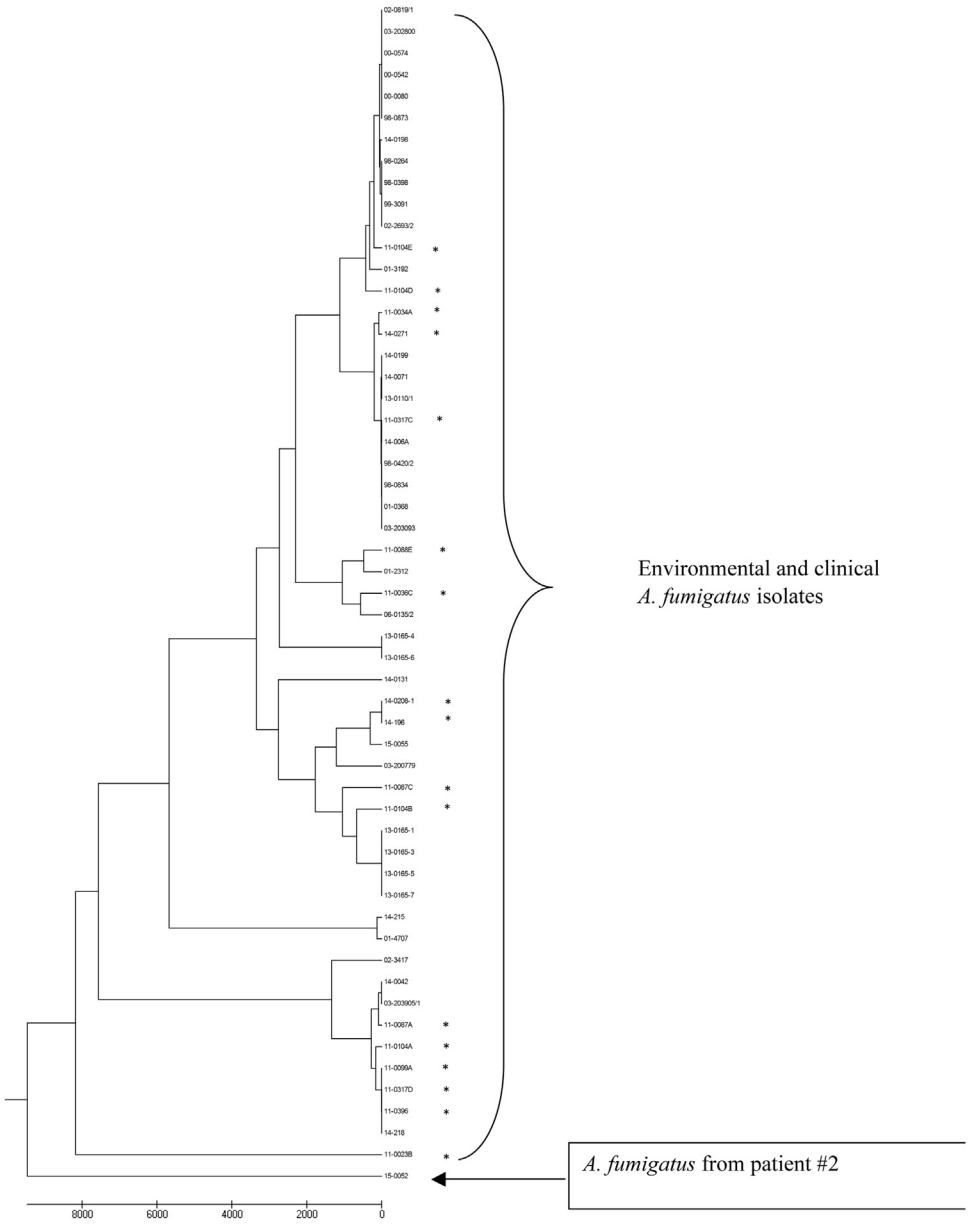

Fig. 2. Dendrogram from unweighted pair group method using arithmetic averages (UPGMA) clustering method based on Nei's genetic distance matrix for *Aspergillus* spp. isolates genotyped at six SSR loci using the environmental and clinical azole-resistant *A. fumigatus* isolates from the same geographical area. *Environmental isolate.

decreased to 0.79% considering only those with *A. fumigatus* isolation. These results from Italy are similar to those of a retrospective study conducted in a haematology department in the Netherlands, where the triazole resistance rate was 1.2% [28] or from a more recent German multicentre surveillance study that reported 1.1% of episodes due to triazole-resistant *A. fumigatus* among acute leukaemia patients [29].

Conversely, a higher rate has been reported in studies carried out in haematology patients in Belgium (4.4%) [30], in the same Netherlands study (27.9%) [18] and in Germany (29.6%) [31]. In Italy, high azole resistance rates were previously reported in different patient groups, such as cystic fibrosis patients (8.2%) [32], or in two studies of a heterogeneous population of patients, 6.25% and 2%, respectively [19,33].

As previously observed by Lestrade et al. [28], the varying incidence rates for azole-resistant *A. fumigatus* might reflect a true variation but may be as a result of differences in methodology—namely, the ability to detect resistance relied on culture and the dominator that was used. To improve the detection of resistance, the analysis of up to five distinct colonies is recommended so as not to miss those that are triazole-resistant [34]. Unfortunately, this more suitable practice was not always applicable because of different guidelines adopted in the participating laboratories.

In addition, we observed the absence of a common protocol of mycological surveillance for haematological patients among the different hospitals participating in this study. Furthermore, the low rate of *Aspergillus* in culture reported from some participating centres may be due to prompt antifungal treatment following a diagnosis of IA through indirect methods. All these factors—indirect diagnosis, no surveillance, analysis of only one colony—could have led us to underestimate triazole resistance.

The environmental origin of *A. fumigatus* triazole resistance, linked to the TR_{34}/L98H mutation, is now shared in the scientific community and this mutation was found in different crops all over the Italian territory [12]. Contrary to results of our previous studies [19,32], in this survey no homology was highlighted by genetic

analysis among the *A. fumigatus sensu stricto* strain and the environmental or clinical strains previously isolated in the same area. However, this observation is certainly limited by the analysis of only a single isolate.

Finally, in the present study, we identified two cryptic triazole-resistant species, one *A. lentulus* (section *Fumigati*), intrinsically azole-resistant, and one *A. awamori* (section *Nigri*). This finding highlights the importance of correct identification at the species level to reach a rapid therapeutic decision and to understand the shift towards other cryptic species quickly.

In conclusion, dedicated surveillance programs should be put in place to have updated information on the local epidemiology and consideration of the high rates of azole resistance in the local environment.

Ethical approval

This was approved by the Ethics Committee of the Università degli Studi di Milano (No. 44/15).

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Conflict of interests

A. Candoni: Gilead: Honoraria, speakers bureau.

C. Fontana: congress lecture fees from Copan Italia and Alifax; research grant from Alifax; Faculty grant from Micon Edra, DotCom; research grant by Quintiles/Angelini; advisory Board: Angelini.

M. Mikulska: speaker and advisory board fees from Pfizer, Janssen Biotest, Gilead and MSD.

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