Voriconazole resistance and mortality in invasive aspergillosis: A multicenter retrospective cohort study

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Als we wisten wat we deden, heette het geen onderzoek. (A. Einstein)

ABSTRACT

Background

Triazole resistance is an increasing problem in invasive aspergillosis (IA). Small case series show mortality rates of 50%-100% in patients infected with a triazole-resistant *Aspergillus fumigatus*, but a direct comparison with triazole-susceptible IA is lacking.

Methods

A 5-year retrospective cohort study (2011-2015) was conducted to compare mortality in patients with voriconazole-susceptible and voriconazole-resistant IA. *A. fumigatus* culture-positive patients in three University Medical Centers were investigated to identify patients with proven, probable and putative IA. Clinical characteristics, day-42 and day-90 mortality, triazole resistance profiles and antifungal treatments were investigated.

Results

Of 196 patients with IA, 37 (19%) harbored a voriconazole-resistant infection. Hematological malignancy was the underlying disease in 103 (53%) patients, and 154 (79%) patients were started on voriconazole. Compared with voriconazole-susceptible cases, voriconazole resistance was associated with an increase in overall mortality of 21% on day-42 (49% versus 28%, p=0.017) and 25% on day-90 (62% versus 37%, p=0.0038) corresponding with a hazard ratio of 1·4 (day-42 95%CI 0.8 to 2.5; p=0.272). In non-ICU patients a 19% lower survival rate was observed in voriconazole-resistant cases at day-42 (p=0.045). The mortality in patients receiving appropriate initial voriconazole therapy was 24% compared with 47% in those receiving inappropriate therapy (p=0.016), despite switching to appropriate antifungal therapy after a median of 10 days.

Conclusions

Voriconazole resistance was associated with an excess overall mortality of 21% at day-42 and 25% at day-90 in patients with IA. A delay in the initiation of appropriate antifungal therapy was associated with increased overall mortality.

Triazoles are the mainstay of therapy for invasive aspergillosis (IA) and have led to a substantial improvement in overall survival. However, triazole resistance has become a concern for the management of infections caused by *Aspergillus fumigatus*. Through culture-based surveillance studies the number of countries that report azole resistance continues to increase, although resistance frequencies vary considerably between different geographic regions [1]. Resistance rates as high as 29% have been observed in specific patient populations, such as critically-ill patients [2]. Variations in resistance

frequencies may reflect true geographical differences or might be due to other variables, including study design, patient populations and laboratory practices [3,4].

Triazole resistance may develop through therapy of individual patients with *Aspergillus* disease primarily occurring in patients with chronic pulmonary aspergillosis [5]. More important, triazole resistance may develop in the environment following exposure to azole fungicides [6]. Patients inhale *A. fumigatus* spores resistant to medical triazoles, which may evolve into triazole-resistant IA. The environmental route is characterized by an apparent lack of patient risk factors as the majority of patients who present with triazole-resistant IA have not been previously treated with medical triazoles [7]. The optimal management of patients suspected of IA in regions with environmental resistance remains unclear, and an expert panel recommend considering moving away from triazole monotherapy when regional resistance frequencies exceed 10% [8]. This 10% threshold has been the subject of debate given the toxicity, costs, lack of oral formulations and of comparative clinical trials of non-triazole antifungals like liposomal amphotericin B (L-AmB) and echinocandins or antifungal combination therapies. Animal experiments consistently show that the efficacy of triazoles in infection with *A. fumigatus* with elevated triazole MICs is reduced compared with wild-type infection [9,10]. This has been shown for itraconazole, voriconazole, posaconazole and isavuconazole. Furthermore, several small case series reported mortality rates of 50%-100% in patients with triazole-resistant IA [11]. These rates are higher than those reported in recent clinical trials, where mortality rates in triazole-treated aspergillosis patients were below 30%, but selection bias may partially explain the very high mortality and therefore, the exact impact of triazole resistance remains to be defined as direct comparisons between triazole-susceptible and triazole-resistant infection are lacking [7,12]. To investigate the characteristics and outcome of voriconazole-susceptible IA and voriconazole-resistant IA, we conducted a retrospective, multicentre study in a large cohort of *A. fumigatus* culture-positive patients.

METHODS

Study design

A retrospective cohort study was performed in three tertiary care University Medical Centers in the Netherlands: Radboud University Medical Center in Nijmegen, Leiden University Medical Center in Leiden and Erasmus University Medical Center in Rotterdam.

General management of IA

Diagnostic work-up in patients suspected of invasive pulmonary mould disease, typically included chest CT and, if possible, bronchoscopy and bronchoalveolar lavage (BAL). In patients with acute myeloid leukemia, myelodysplastic syndrome, and hematopoietic stem cell transplant recipients a diagnostic driven strategy was used including monitoring of serum galactomannan (GM) during neutropenia or in febrile patients. Chest CT was performed in patients with positive serum GM, in those with persistent fever despite three to five days of broad-spectrum antibacterial therapy, and in patients with progressive respiratory failure. If CT confirmed the presence of pulmonary infiltrates, a BAL was performed for fungal culture and GM measurement. Voriconazole was the first choice treatment option for patients with IA. During the study period no hospital treatment guidelines were available for documented voriconazole-resistant IA, but when resistance was documented or suspected in critically-ill patients, treatment was changed to either triazole and echinocandin combination therapy or L-AmB.

Data collection

The microbiology database was searched for positive *A. fumigatus* cultures of patients admitted between January 2011 and December 2015. In order to select patients with IA the clinical records of culture-positive patients needed to meet three conditions: (i) antifungal therapy was started within one month before or after a positive culture, (ii) the patient had received at least two days of antifungal therapy, and (iii) the patient could be classified as probable or proven IA according to EORTC/MSG definitions or putative or proven according to AspICU criteria for the subgroup of patients admitted to the ICU [13,14].

Patient characteristics included age, gender, underlying diseases, ward/ICU admission, and antifungal prophylaxis or therapy. ICU admission was defined as initiation of antifungal therapy in the ICU and stay in the ICU for at least two consecutive days. In addition, patients who were admitted to the ICU during their hospitalization were analyzed separately in a cox regression model. Furthermore, the appropriateness of initial antifungal therapy was assessed for patients treated with voriconazole. Antifungal therapy was considered appropriate if voriconazole was started in patients with

voriconazole-susceptible disease and inappropriate in those with voriconazole-resistant IA. Switch to appropriate antifungal therapy and time to switch were determined.

The study was reviewed by the Institutional Review Boards, which confirmed that the study did not fall under the Dutch law on research on human subjects. Data were processed after encoding and in accordance with the Dutch Personal Data protection act.

Mycology

Fungal cultures were routinely performed if a patient underwent bronchoscopy with BAL and if ordered for other respiratory specimens. *A. fumigatus* was identified by macroscopic and microscopic morphology and growth at 48°C. *A. fumigatus* isolates were routinely screened for the presence of triazole resistance using an agar-dilution method (VIPcheck™, Nijmegen, the Netherlands) [15]. The method relies on agar-wells supplemented with itraconazole, voriconazole and posaconazole and a growth control. Fungal growth on any triazole-containing well was considered indicative of resistance and these isolates were sent to Radboud University Medical Center for MIC-testing according to the EUCAST reference method [16]. Infection was considered to be voriconazole-resistant if one or more cultured *A. fumigatus* isolates exhibited a voriconazole MIC above the clinical breakpoint of 2 mg/l. If a patient had more isolates cultured within one month of initiation of antifungal therapy, the most resistant isolate was used to classify the patient. In addition, the presence of a resistance mutation in cyp51A was determined by *Cyp51A*-gene sequencing, which is specific for *A. fumigatus* sensu strictu, excluding sibling species from the *A. fumigatus* species complex [17,18].

Data analysis

The primary endpoints were day-42 and day-90 mortality in voriconazole-resistant IA compared with voriconazole-susceptible IA cases. Day zero was set at day of initiation of antifungal therapy. Other factors with possible impact on survival were also investigated including choice of first-line antifungal therapy, ICU-admission, Acute Physiology And Chronic Health Evaluation II (Apache II) score, and appropriateness of initial antifungal therapy.

Statistical analysis

Statistical analysis on the relation of voriconazole resistance and mortality was performed in SAS® 9.4 and SPSS® 24 with survival analysis (Kaplan-Meier) and the log-rank method. Confidence intervals were calculated with the Kaplan-Meier method. Possible confounders, i.e. ICU admission, underlying hematological disease and center, were analyzed for each comparison with Cox regression survival analysis, Kaplan-Meier survival (Log rank) and Fisher exact. Other differences were compared with Fisher exact.

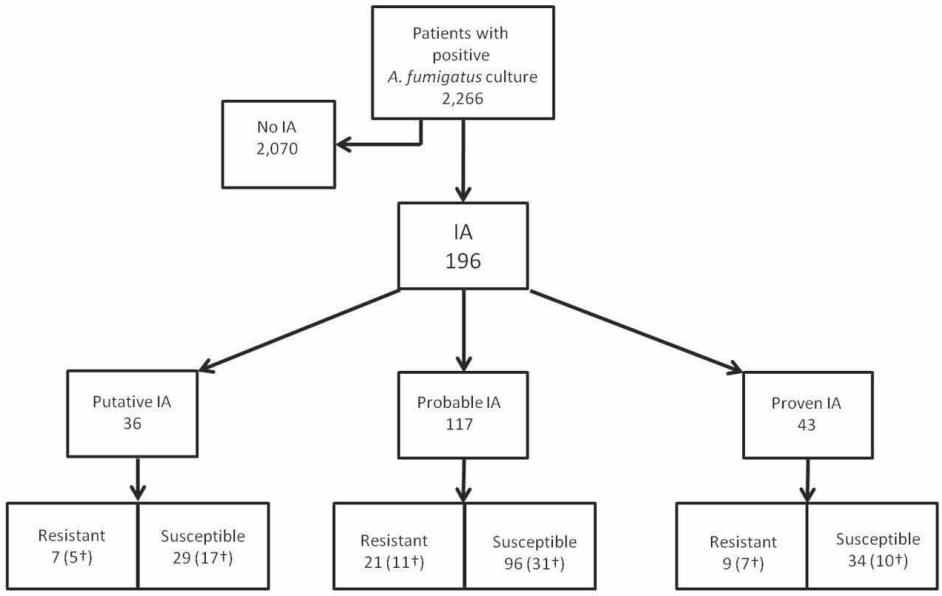

Figure 1: Inclusion of patients with positive *A. fumigatus* cultures from lower respiratory tract or sterile specimens between January 2011 and December 2015. 2,266 patients had one or more positive cultures, and 196 patients could be classified according to the definitions of AspICU and EORTC/MSG. IA, invasive aspergillosis; †, mortality.

RESULTS

Demographics

In the five-year period 2,266 patients with a positive *A. fumigatus* culture in the three centers were eligible for the study. Overall, 196 (8.6%) patients met our case definition, i.e. received antifungal therapy within 30 days of a positive culture, received at least two days of antifungal therapy and could be classified according to the EORTC/MSG or AspICU criteria (Figure 1). A proven infection was documented in 43 (22%) patients, a putative diagnosis in 36 (18%) patients and a probable diagnosis in 117 (60%) patients (Table 1). Hematological malignancy was the most frequent underlying disease diagnosed in 103 of 196 (53%) patients. Eighty-five (43%) patients were admitted to the ICU during hospital admission, while 59 (30%) patients first received antifungal therapy in the ICU. Voriconazole was the initial therapy in 154 (79%) patients. Further details regarding the demography for individual centers and the total patient population are shown in Table 1.

Voriconazole susceptibility

Voriconazole resistance was observed in 37 of 196 (19%) patients, but the resistance frequency varied between 10% and 31% in individual centers (Table 1). Voriconazole-

Parameter[a]		Center 1 (60 cases)	Center 2 (59 cases)	Center 3 (77 cases)	Total (196 cases)
Patient	Male / Female	34 / 26	36 / 23	45 / 32	115 / 81
	Median age	64 (3 - 79)	61 (4 - 80)	61 (2 - 83)	62 (2-83)
	Hematological malignancy	23 (38%)	34 (58%)	46 (60%)	103 (53%)
	Autoimmune disease	12 (20%)	6 (10%)	6 (8%)	24 (12%)
	Solid organ transplant	8 (13%)	5 (8%)	11 (14%)	24 (12%)
	Structural lung disease	5 (8%)	6 (3%)	3 (4%)	14 (7%)
	Solid tumor	3 (5%)	-	3 (4%)	6 (3%)
	Congenital immune disorder	4 (7%)	-	-	4 (2%)
	Other	4 (7%)	8 (4%)	2 (3%)	14 (7%)
	None	1 (2%)	-	6 (8%)	7 (4%)
	ICU-admission	20 (33%)	23 (39%)	16 (21%)	59 (30%)
	APACHE II-score	21	25	21	22
IA	Putative	15 (25%)	14 (24%)	7 (9%)	36 (18%)
	Probable	24 (40%)	39 (66%)	54 (70%)	117 (60%)
	Proven	21 (35%)	6 (10%)	16 (21%)	43 (22%)
	Voriconazole-susceptible	54 (90%)	41 (69%)	64 (83%)	159 (81%)
	Voriconazole-resistant	6 (10%)	18 (31%)	13 (17%)	37 (19%)
ICU	Voriconazole-susceptible	18 (90%)	17 (74%)	10 (63%)	45 (76%)
	Voriconazole-resistant	2 (10%)	6 (26%)	6 (37%)	14 (24%)
non-ICU	Voriconazole-susceptible	36 (90%)	24 (67%)	54 (89%)	114 (83%)
	Voriconazole-resistant	4 (10%)	12 (33%)	7 (11%)	23 (17%)
Management	Triazole prophylaxis	-	16 (27%)	4 (5%)	20 (10%)
	Voriconazole	42 (70%)	45 (76%)	67 (87%)	154 (79%)
	Itraconazole	1 (2%)	-	-	1 (0.5%)
	Posaconazole	1 (2%)	-	2 (3%)	3 (2%)
	L-AmB	13 (22%)	13 (22%)	1 (1%)	27 (14%)
	Echinocandin	-	-	1 (1%)	1 (0.5%)
	VCZ+L-AmB	-	-	4 (5%)	4 (2%)
	VCZ+Ecand	3 (5%)	1 (2%)	1 (1%)	5 (3%)
	VCZ+intrathecal caspofungin/L-AmB	-	-	1 (1%)	1 (0.5%)
	Inappropriate therapy / appropriate therapy	3 / 57	15 / 44	12 / 65	30 /196
Outcome	42-day mortality	13 (22%)	26 (65%)	23 (30%)	62 (32%)
	90-day mortality	23 (38%)	29 (85%)	29 (38%)	81 (42%)
	42-day mortality ICU	11 (55%)	18 (78%)	7 (44%)	36 (61%)
	90-day mortality ICU	13 (65%)	20 (87%)	7 (44%)	40 (68%)
	42-day mortality non-ICU	2 (3%)	8 (14%)	15 (19%)	25 (18%)
	90-day mortality non-ICU	10 (17%)	9 (15%)	22 (29%)	41 (30%)

Table 1: Demographics, invasive aspergillosis classification, voriconazole susceptibility, management and outcome of 196 patients with culture-positive invasive aspergillosis.
[a]L-AmB, liposomal-amphotericin B; VCZ, voriconazole.

resistant IA was diagnosed in 14 of 59 (24%) ICU patients and in 23 of 137 (17%) non-ICU patients. Voriconazole resistance corresponded with resistance to isavuconazole for all 14 patients where isavuconazole susceptibility was determined (Table 2). In 30 of 37 (81%) patients the *A. fumigatus* isolate showed a pan-triazole resistant phenotype, while in seven patients the susceptibility to itraconazole, voriconazole and posaconazole varied. Analysis of triazole-resistant *A. fumigatus* isolates showed resistance mutations that are associated with the environmental route of resistance selection in 32 of 37 (87%) patients; TR_{34}/L98H in 18 patients and TR_{46}/Y121F/T289A in 13 patients (Table 2). In five voriconazole-resistant isolates no mutations were found in the *Cyp51A*-gene, suggesting that other uncharacterized resistance mutations might be present. In seven patients (19%) a mixed infection was diagnosed; this consisted of an infection with a triazole-resistant and susceptible *A. fumigatus* in six patients (S/R), while in one patient isolates with two different resistance mutations were recovered (R/R; Table 2). All cultured *A. fumigatus* isolates were susceptible to amphotericin B.

Mortality

The overall mortality in the 195 patients with IA was 62 (32%) at day-42 and 81 (42%) at day-90 (Table 1). One patient was discharged to a hospice on day-25 but the exact day of death was not known, therefore his survival was censored at 25 days. Comparing the patients infected with voriconazole-susceptible and voriconazole-resistant *A. fumigatus*, a 21% higher overall mortality was observed in patients infected with a resistant isolate; 44 of 158 (28%; 95%CI 21% to 35%) patients with voriconazole-susceptible infection had died at day-42 versus 18 of 37 (49%; 95%CI 34% to 66%; log-rank test, p=0.017) of those with voriconazole-resistant IA. At day-90 the absolute difference in mortality had increased to 25% (58 of 158; 37%; 95%CI 30% to 45% and 23 of 37; 62%; 95%CI 47% to 77%, respectively; log-rank test, p=0.0038; Figure 2A). As expected, the cumulative survival rates were much lower for 59 patients who first received antifungal therapy in the ICU; mortality was 26 of 45 (58%) for patients with voriconazole-susceptible IA and 10 of 14 (71%) for those with voriconazole-resistant IA at day-42 (log-rank test, p=0.37; Figure 2B). For 136 patients who first received antifungal therapy on the ward (non-ICU group) a 19% lower survival rate was observed for patients with voriconazole-resistant IA, compared with voriconazole-susceptible infection, with a mortality rate of 8 of 23 (35%) and 19 of 113 (16%) at day-42, respectively (log-rank test, p=0.045; Figure 2C). The mortality for 18 patients infected with TR_{34}/L98H was not different from 13 patients with TR_{46}/Y121F/T289A infection (supplementary Figure S1).

At the discretion of the treating physician, 27 of 196 (14%) patients received initial antifungal therapy with L-AmB. Eight of these 27 patients were infected with voriconazole-resistant *A. fumigatus*. The survival at day-42 of L-AmB treated patients was 55% compared with 71% for voriconazole-treated patients (log-rank test, p=0.04; Figure 3).

Sex, age	Center	Underlying disease[¤]	ICU	IA classification	Sample with resistant culture	Cyp51A-resistance mutation[¥]	MIC (mg/l)[¤]				Initial antifungal therapy[§]	Outcome at day 90 (day of death)
M, 49	1	Diabetes, necrotizing pancreatitis	Yes	Proven	Lung biopsy	TR_{34}/L98H	>16	16	1	8	L-AmB	Alive
M, 71	1	Kidney transplant	No	Proven	Bronchial aspirate	TR_{46}/Y121F/T289A$^{*S/R}$	0.5	>16	0.25	>16	VCZ	Died (+50)
M, 55	1	AML, alloHSCT	No	Probable	Sputum	WT	1	4	0.25	-	L-AmB	Died (+52)
M, 70	1	COPD, lung fibrosis	Yes	Putative	BAL	WT	>16	>16	1	-	VCZ	Died (+14)
F, 59	1	T cell lymphoma	No	Proven	Sinus biopsy	TR_{34}/L98H	>16	4	0.5	-	L-AmB	Died (+15)
F, 65	1	Kidney transplant	No	Proven	Brain biopsy	TR_{34}/L98H$^{*S/R}$	>16	8	0.5	8	VCZ	Died (+90)
F, 69	2	MDS	Yes	Probable	BAL	TR_{46}/Y121F/T289A	>16	>16	1	-	L-AmB	Alive
M, 51	2	B cell lymphoma	No	Probable	Sputum	TR_{34}/L98H	>16	8	0.5	-	VCZ	Alive
F, 64	2	B cell lymphoma	Yes	Probable	BAL	TR_{46}/Y121F/T289A	>16	>16	1	-	L-AmB	Died (+25)
M, 59	2	AML	Yes	Putative	BAL	TR_{34}/L98H	16	8	1	-	VCZ	Died (+27)
F, 39	2	Liver transplant	Yes	Putative	BAL	TR_{34}/L98H	>16	16	1	-	VCZ	Died (+62)
F, 44	2	Auto immune hepatitis	Yes	Putative	BAL	TR_{46}/Y121F/T289A	>16	>16	1	-	VCZ	Died (+7)
M, 61	2	B cell lymphoma	No	Probable	Sputum	TR_{46}/Y121F/T289A$^{*R/R}$	>16	>16	1	-	L-AmB	Died (+11)
					BAL	TR_{34}/L98H	>16	8	1	-		
M, 15	2	Severe aplastic anaemia	Yes	Probable	BAL	TR_{34}/L98H	>16	8	1	-	L-AmB	Died (+4)
F, 53	2	Lung carcinoma	Yes	Probable	Drain fluid	TR_{34}/L98H	>16	4	0.5	-	VCZ	Died (+7)
M, 67	2	COPD	Yes	Proven	Lung autopsy	TR_{34}/L98H	>16	8	1	-	VCZ	Died (+8)
F, 19	2	B cell lymphoma	No	Probable	Sputum	TR_{34}/L98H	>16	8	1	16	VCZ	Died (+18)
M, 54	2	B cell lymphoma	No	Probable	BAL	TR_{34}/L98H$^{*S/R}$	>16	8	1	8	VCZ	Alive
M, 45	2	B cell lymphoma	No	Probable	Bronchial aspirate	TR_{46}/Y121F/T289A$^{*S/R}$	1	>16	0.25	>16	VCZ	Alive
F, 67	2	AML	No	Probable	Bronchial aspirate	TR_{46}/Y121F/T289A	>16	>16	0.25	>16	VCZ	Alive
M, 62	2	B cell lymphoma, alloHSCT	No	Proven	Tissue biopsy	TR_{46}/Y121F/T289A	0.5	>16	0.5	>16	VCZ	Alive
M, 71	2	Influenza	Yes	Putative	Sputum	TR_{34}/L98H$^{*S/R}$	>16	>16	2	>16	VCZ	Alive
M, 70	2	AML	Yes	Probable	Bronchial aspirate	TR_{34}/L98H	>16	8	1	8	VCZ	Alive

Sex, age	Center	Underlying disease[¤]	ICU	IA classification	Sample with resistant culture	Cyp51A-resistance mutation[¥]	MIC (mg/l)[¤]				Initial antifungal therapy[§]	Outcome at day 90 (day of death)
F, 58	2	Follicular lymphoma	Yes	Putative	BAL	WT[*S/R]	>16	>16	1	>16	VCZ	Alive
M, 22	3	alloHSCT	Yes	Probable	Sputum	TR_{46}/Y121F/T289A	>16	>16	1	-	L-AmB	Died (+9)
M, 46	3	Kidney Tx, Marginal zone lymphoma	No	Probable	BAL	TR_{34}/L98H	>16	4	0.5	8	VCZ	Alive
M, 53	3	Haematological malignancy	No	Probable	Sputum	TR_{46}/Y121F/T289A	>16	>16	1	-	VCZ	Died (+66)
F, 64	3	Haematological malignancy	No	Probable	Sputum	TR_{34}/L98H	>16	16	2	-	VCZ	Alive
M, 64	3	Haematological malignancy	No	Probable	BAL	TR_{46}/Y121F/T289A	>16	4	1	-	VCZ	Alive
M, 29	3	Hodgkin lymphoma, alloHSCT	Yes	Probable	BAL	TR_{34}/L98H	>16	8	2	-	VCZ	Died (+44)
F, 63	3	Lung transplant	Yes	Probable	Sputum	TR_{34}/L98H	>16	16	2	16	VCZ	Died (+40)
F, 64	3	Neurosarcoidosis	Yes	Probable	Sputum	WT	0.25	4	0.25	-	VCZ	Died (+4)
F, 40	3	alloHSCT	Yes	Proven	BAL	TR_{34}/L98H	>16	4	1	-	VCZ	Died (+32)
M, 56	3	Autoimmune disease	Yes	Proven	Sputum	TR_{46}/Y121F/T289A	2	>16	1	-	VCZ	Died (+32)
F, 60	3	SOT	Yes	Proven	Sputum	TR_{46}/Y121F/T289A	16	>16	0.5	-	VCZ	Alive
F, 62	3	Hematological malignancy, influenza	Yes	Proven	BAL	TR_{46}/Y121F/T289A	2	>16	1	-	VCZ	Died (+10)
M, 38	3	Poly trauma	Yes	Putative	BAL	WT	>16	4	0.5	8	VCZ	Died (+24)

Table 2: Underlying condition, invasive aspergillosis classification, *A. fumigatus* genotype and phenotype, and outcome in 37 patients with voriconazole-resistant IA.

[¤]AML, acute myeloid leukaemia; AlloHSCT, allogeneic haematopoietic stem cell transplant; COPD, chronic obstructive pulmonary disease; MDS, myelodysplastic syndrome; CLL, chronic lymphoblastic laeukemia; SOT, solid organ transplant.

[¥] WT, wild type.

*Mixed infection; [*S/R] voriconazole-susceptible and voriconazole-resistant; [*R/R] two different voriconazole-resistant genotypes.

[¤]ITZ, itraconazole, VCZ, voriconazole; POS, posaconazole; ISA, isavuconazole.

[§]VCZ, voriconazole, L-AmB, liposomal amphotericin B.

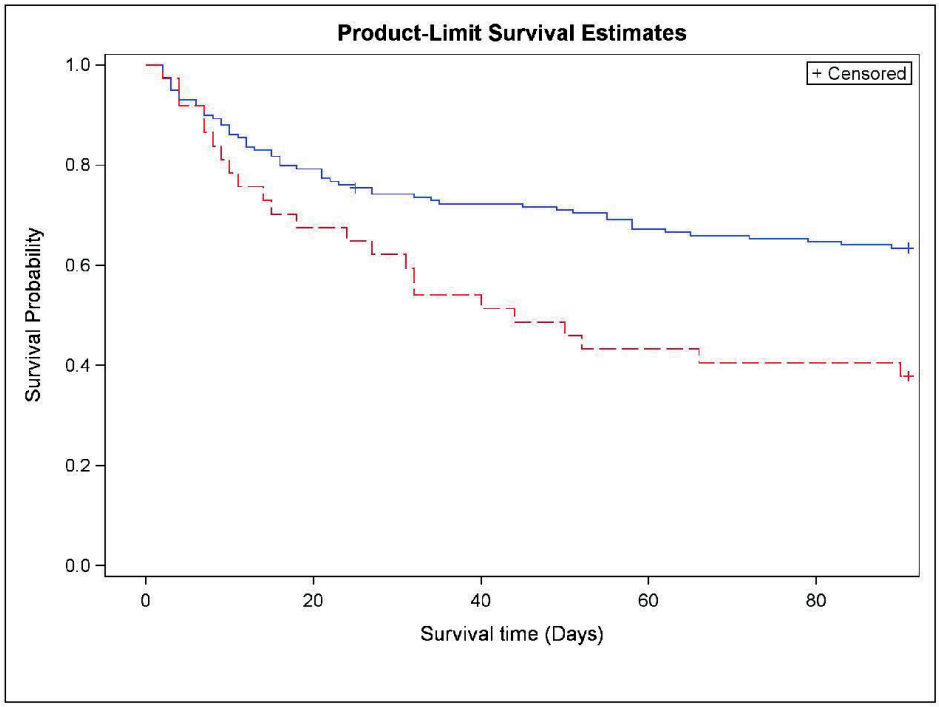

Figure 2A

Group / Day	0	42		90	
VCZ-S	159	114	(72%; CI 65% - 79%)	100	(63%; CI 55% - 70%)
VCZ-R	37	19	(51%; CI 34% - 66%)	14	(38%; CI 23% - 53%)
P-value log rank test			0.017		0.0038

Figure 2: Cumulative survival of patients with voriconazole-susceptible and voriconazole-resistant IA. A. Cumulative survival of all patients with IA. B. Cumulative survival of patients that started antifungal therapy at the ICU. C. Cumulative survival in non-ICU patients with IA. Blue lines represent patients with IA due to voriconazole-susceptible *A. fumigatus* (VCZ-S); Red lines represent patients with IA due to voriconazole-resistant *A. fumigatus* (VCZ-R). One patient was discharged to a hospice after 25 days and his survival was therefore censored at day-25.

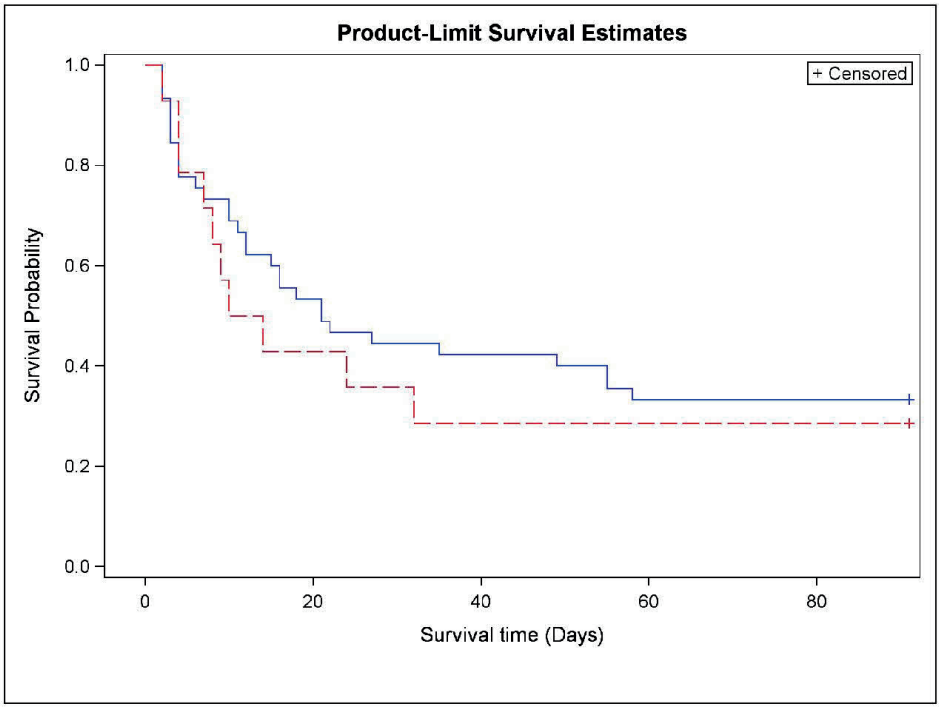

Figure 2B

Group / Day	0	42		90	
VCZ-S	45	19	(42%; CI 28% - 56%)	15	(33%; CI 20% - 47%)
VCZ-R	14	4	(29%; CI 9% - 52%)	4	(29%; CI 9% - 52%)
P-value log rank test			0.37		0.57

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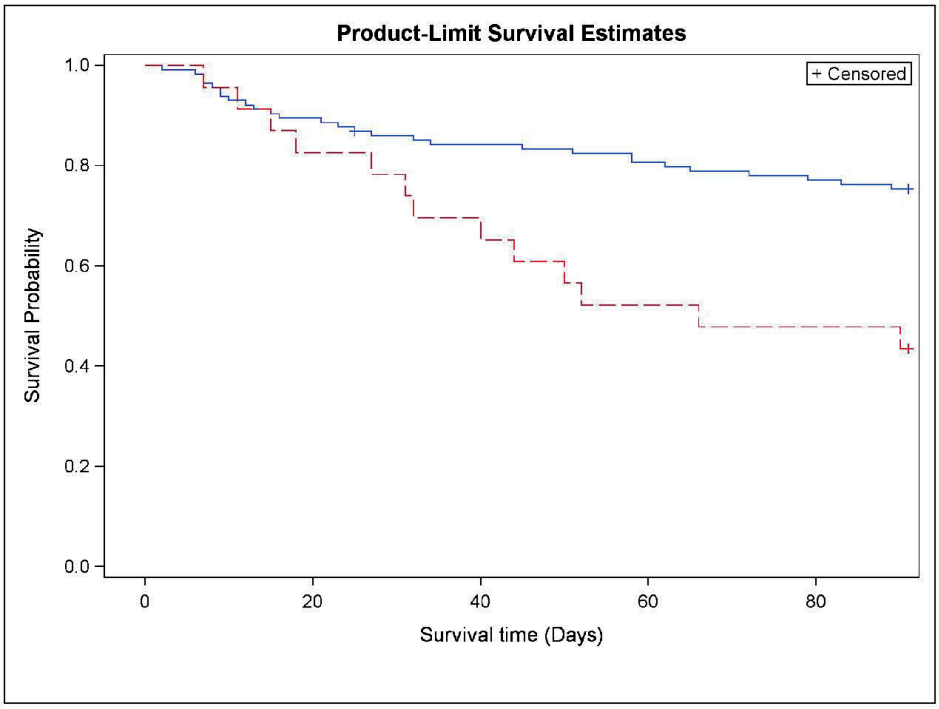
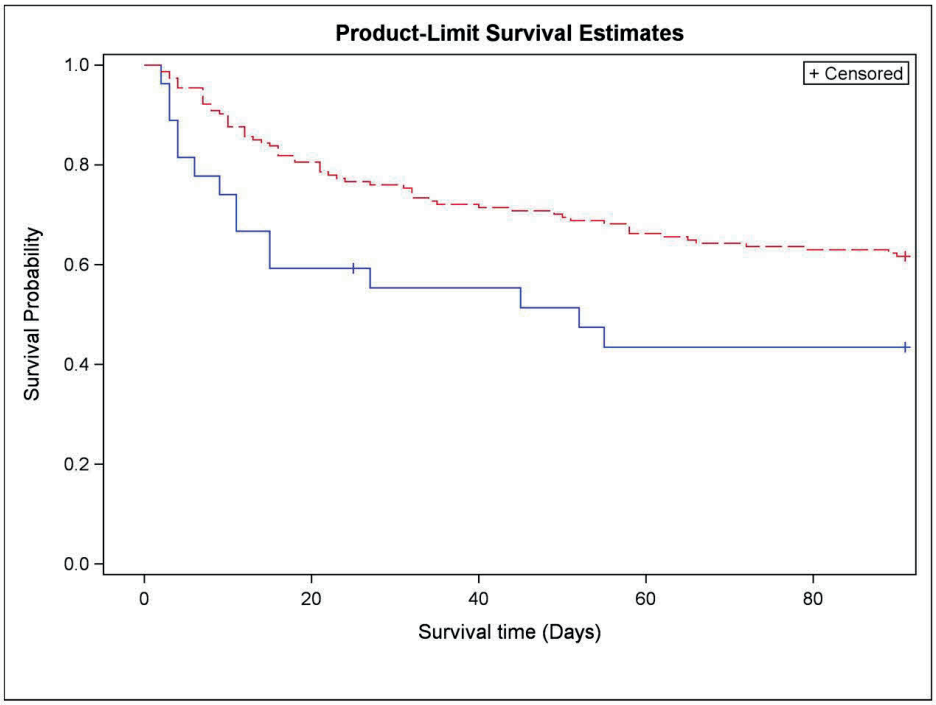


Figure 2C

Group / Day	0	42		90	
VCZ-S	114	95	(84%; CI 76% - 90%)	85	(75%; CI 66% - 82%)
VCZ-R	23	15	(65%; CI 42% - 81%)	10	(43%; CI 23% - 62%)
P-value log rank test			0.045		0.002

Figure 2: Cumulative survival of patients with voriconazole-susceptible and voriconazole-resistant IA. A. Cumulative survival of all patients with IA. B. Cumulative survival of patients that started antifungal therapy at the ICU. C. Cumulative survival in non-ICU patients with IA. Blue lines represent patients with IA due to voriconazole-susceptible *A. fumigatus* (VCZ-S); Red lines represent patients with IA due to voriconazole-resistant *A. fumigatus* (VCZ-R). One patient was discharged to a hospice after 25 days and his survival was therefore censored at day-25.

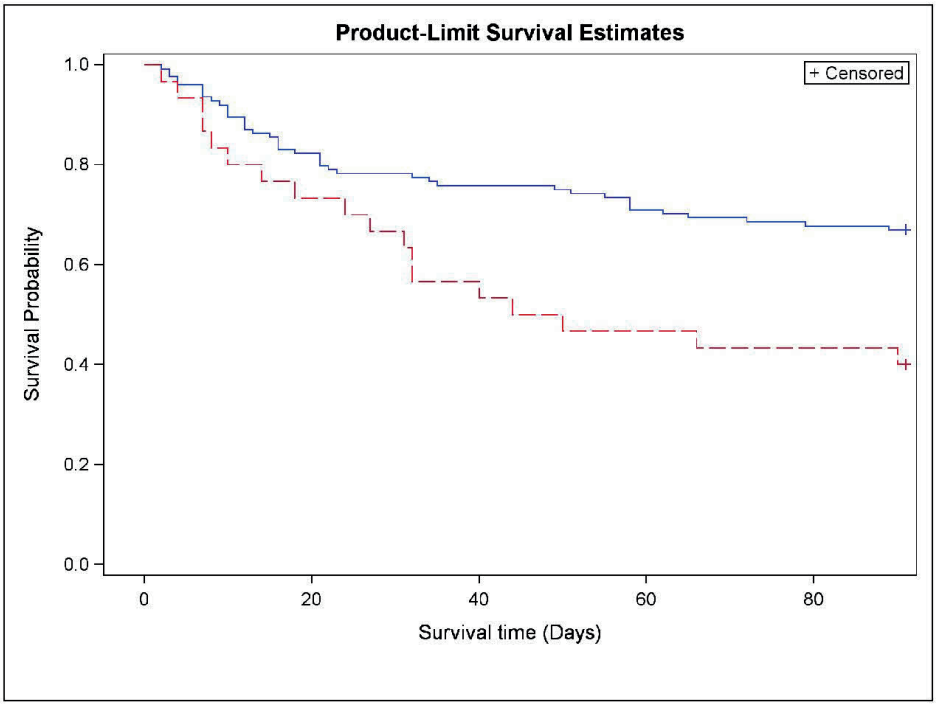


Group / Day	0	42		90	
VCZ	154	110	(71%; CI 64% - 78%)	95	(62%; CI 54% - 69%)
L-AmB	27	14	(55%; CI 35% - 72%)	11	(43%; CI 24% - 61%)
P-value log rank test			0.04		0.025

Figure 3: Cumulative survival of L-AmB-treated IA patients compared with voriconazole-treated patients. Red line represents patients with IA who were treated with voriconazole (VCZ); Blue line represents patients with IA who were treated with liposomal amphotericin B (L-AmB). One patient was discharged to a hospice after 25 days and his survival was therefore censored at day-25.

However, the proportion of ICU-patients in the L-AmB-treated group was significantly higher compared with voriconazole-treated patients (13 of 27 (48%) versus 43 of 154 (28%); Fisher exact test, p=0.04), indicating that confounding by indication at least partly explained this difference.

The mortality of patients receiving appropriate and inappropriate therapy was compared for 154 patients with initial voriconazole therapy. Thirty patients (81%) with voriconazole-resistant IA initially received voriconazole therapy and were classified to have received inappropriate antifungal therapy (Table 2). Therapy was switched to appropriate therapy in 18 patients after a median of 10 days (range 1 to 39 days). Inappropriate voriconazole therapy corresponded with reduced survival at day-42 compared with appropriate therapy (76% and 53%, respectively; log-rank test, p=0.016; Figure 4). Six patients presented with mixed infection (Table 2).



Group / Day	0	42		90	
Appropriate	124	94	(76%; CI 67% - 82%)	83	(67%; CI 58% - 74%)
Inappropriate	30	16	(53%; CI 34% - 69%)	12	(40%; CI 23% - 57%)
P-value log rank test			0.016		0.0049

Figure 4: Cumulative survival of patients that initially received voriconazole therapy; patients receiving appropriate initial voriconazole therapy were compared with those receiving inappropriate therapy. Blue line represents patients with IA who received appropriate initial voriconazole therapy; red line represents patients with IA who received inappropriate initial voriconazole therapy.

Cox regression analysis

ICU admission, underlying hematological disease, and center were analyzed as possible confounders for mortality. ICU admission contributed significantly to mortality, whereas the presence of hematological disease had no effect (see Supplementary Table 1). Comparison of the centers indicated that the resistance frequency was significantly higher in centre 2 in comparison with centers 1 and 3 (p=0.009). The hazard ratio at day-42 for patients who started voriconazole therapy on the ICU was 7.7 (95%CI 3.9 to 15.3; p<0·001), while a hazard ratio of 1.4 was found for voriconazole resistance (95%CI 0.8 to 2.4; p=0·272; Table S1). In patients where voriconazole therapy was initiated on the ward, voriconazole resistance frequency was higher in patients who required ICU-admission compared to those who completed treatment on the ward (8 of 16 (50%) compared with 15 of 111 (14%) patients, respectively; p=0.044).

DISCUSSION

Our retrospective cohort study showed a higher mortality in patients with voriconazole-resistant IA compared with voriconazole-susceptible IA. In a setting of primary therapy with voriconazole, the absolute difference in day-42 and day-90 mortality ranged between 21% and 33%, respectively for the overall patient group and for non-ICU patients. These observations are in line with results from in-vivo models of resistant infection and case series [7,9,10,12,19]. However, these case series included a small number of IA patients and were therefore prone to selection or publication bias. In the subset of patients admitted to the ICU, no significant difference in survival between voriconazole-resistant and voriconazole-susceptible IA was found. However, the smaller sample size of this subgroup as well as the high mortality of 67% in voriconazole-susceptible IA patients in the ICU makes this analysis severely underpowered.

L-AmB is considered alternative treatment for IA but a randomized comparison with voriconazole has never been performed and therefore, its efficacy relative to voriconazole remains unclear [24]. In our study the survival of L-AmB treated patients was not better than voriconazole-treated patients with IA. However, patients that received L-AmB were more often admitted to the ICU compared with patients on voriconazole and therefore had an a priori higher probability of dying. Although the very small number of patients in this subanalysis makes any definite conclusions premature, this may indicate that in critically-ill patients and those with advanced IA the clinical deterioration could not be reversed by polyene-based therapy. Indeed, pre-clinical studies showed that L-AmB, even at a dose of 10 mg/kg, was ineffective when treatment was delayed until 48 hours post-infection [25], underscoring the need for early intervention. Treatment delay was also found to be associated with poorer outcome of IA in clinical studies [26], which is supported by our observation of lower survival when the initial antifungal therapy was inappropriate.

Voriconazole resistance was dominated by mutations associated with environmental resistance selection, accounting for 87% of resistance mutations [7,11,12]. The majority of isolates were pan-azole resistant and there was 100% cross-resistance between voriconazole and isavuconazole. There are no known risk factors that can help to identify patients at high risk for triazole-resistant IA, and in our study all cases of inappropriate antifungal therapy were due to voriconazole therapy in voriconazole-resistant IA.

Our study has several limitations, including its retrospective design. Many factors may impact on the outcome of IA and some of these could act as confounder as they may not be well balanced between voriconazole-susceptible and voriconazole-resistant patient groups. We identified possible confounders in our cohort. As expected ICU-admission was associated with significant higher mortality. However, when ICU-patients were excluded, mortality in voriconazole-resistant IA remained significantly higher compared

with voriconazole-susceptible IA. Furthermore, patients with voriconazole-resistant IA were more likely to require ICU-admission, suggesting that initial therapy was not successful. Cox regression analysis indicated that the hazard of death due voriconazole-resistance was 1.4 times higher than in voriconazole-susceptible infection.

Our study relied on aspergillus culture as this enabled reliable resistance screening and in-vitro susceptibility testing. Agar-based resistance screening through VIPcheck™ was found to be highly sensitive and specific to identify resistant *A. fumigatus* colonies in cultures, and unlike PCR-based resistance detection, allows detection of a broad range of resistance mutations, including uncharacterized mechanisms [15]. However, sensitivity of culture is low and thus our cohort represents a small subset of IA cases and may not be directly translatable to culture negative cases of IA. However, a recent study that used PCR cyp51A resistance testing directly on BAL of hematology patients with IA showed a 31% difference in overall mortality, similar to what we observed [19].

As 79% of patients received initial therapy with voriconazole, our study represents an escalation strategy, i.e. initial voriconazole and escalation when resistance is documented. An escalation strategy is recommended by the IDSA, where MIC-testing is advocated in patients suspected of resistance or failing to primary antifungal therapy [24]. In our study a higher mortality was observed if patients with voriconazole-resistant IA started on voriconazole despite intensive resistance screening. Treatment was switched after a median of 10 days, which did not prevent poor clinical outcome. A management strategy based on less intensive resistance testing, such as recommended by the IDSA, might result in excess mortality in those patients with voriconazole-resistant IA. Direct detection of resistance mutations by molecular techniques in BAL-fluid may reduce the time to resistance detection, and PCR-based strategic studies are currently ongoing.

As appropriate initial antifungal therapy was found to be critical, upfront combination antifungal therapy may be required to increase the probability of survival of patients at risk for IA in geographic regions with high resistance rates. Combination therapy includes voriconazole or isavuconazole combined with an echinocandin or L-AmB, but clinical evidence supporting these treatment options is lacking. However, the 10% threshold recommended by an expert panel was met in our centers, and the Dutch treatment guideline has now been revised recommending routine triazole resistance testing and combination therapy for patients suspected of IA, at least until the presence of resistance has been ruled out [27]. In most countries resistance rates are lower than reported in the Netherlands, which does not justify a de-escalation strategy [1,28].

Our findings underscore the need for rapid resistance tests and antifungal drugs based on new targets. As azole fungicide use appears to be an important driver for resistance in *A. fumigatus* and new resistance mutations continue to emerge in the environment [29], strategies need to be developed aimed at overcoming resistance selection in the environment. However, antimicrobial resistance (AMR) action plans and 'One-Health'

research are generally restricted to bacterial resistance [30]. Governments, medical research councils and public health organizations are called to action to prioritize fungal research and help to overcome the problem of triazole resistance.

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Potential conflicts of Interest

P.E.V. has received financial support from Gilead Sciences, MSD, Pfizer, F2G and non-financial support from OLM and IMMY, outside submitted work. B.J.A.R. received financial support from Gilead Sciences, outside submitted work. A.F.A.D.S. has received financial support from Abvie, Roche, Gilead Sciences and Pfizer, outside submitted work.

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