Triazole-resistant *Aspergillus fumigatus* in the Netherlands between 1994 and 2022: a genomic and phenotypic study

Yinggai Song, Jochem B Buil, Johanna Rhodes, Jan Zoll, Marlou Tehupeiory-Kooreman, Mehmet Ergün, Jianhua Zhang, Ruoyu Li, Thijs Bosch, Willem J G Melchers, Paul E Verweij

Summary

Background ***Aspergillus fumigatus*** **is the main cause of invasive aspergillosis and triazole antifungals are the primary treatment option. The effectiveness of triazole therapy is hampered by the emergence of resistance, mainly caused by mutations in the *cyp51A* gene and a tandem repeat (TR) of 34 bases (TR_{34}/Leu98His) and 46 bases (TR_{46}/Tyr121Phe/Thr289Ala) in the promoter region, which correspond with signature triazole resistance phenotypes. We aimed to investigate the occurrence of triazole phenotype and genotype variation over a 29-year period in the Netherlands.**

Methods **In this genomic and phenotypic study, we screened all clinical *A fumigatus* isolates from Dutch hospitals collected between Jan 6, 1994, and Dec 31, 2022, for resistance to triazole using agar-based methods, and characterised them by sequencing the *cyp51A* gene and in vitro susceptibility testing using the European Committee on Antimicrobial Susceptibility Testing reference method. Whole-genome sequencing was performed on selected isolates, including those harboring TR_{34} variants, high-frequency single-nucleotide polymorphisms, and wild-type strains. Clinical information such as age, underlying disease, diagnosis, therapy, and outcomes was collected for patients who had isolates cultured at the Radboud University Medical Centre, Nijmegen, Netherlands, between Jan 1, 2017, and Dec 31, 2022.**

Findings **1979 (15·6%) of the screened 12 679 *A fumigatus* isolates harboured *cyp51A* triazole resistance mutations, predominately TR_{34}/Leu98His sensu stricto in 1338 (67·6%) resistant isolates and TR_{46}/Tyr121Phe/Thr289Ala sensu stricto in 332 (16·8%) resistant isolates. Phenotype and genotype variations were observed in 325 (17·2%) triazole resistant isolates harbouring a TR-resistance mechanism, including 12 *cyp51A* genotype variants. Whole-genome sequencing showed that isolates with combinations of TR_{34}-based and TR_{46}-based polymorphisms seemed to be derived from separate populations, but there was some overlap. 59 cases of proven or probable invasive aspergillosis were identified, including 13 triazole-resistant cases, of which three were caused by genotype variants. Mixed genotype infection was observed in 11 (84·6%) of 13 triazole-resistant patients and the number of antifungal treatment switches was higher compared with triazole-susceptible disease ($p<0·0001$).**

Interpretation **Our study showed variation in triazole genotypes and phenotypes in clinical *A fumigatus* isolates with cyp51A-mediated resistance, some of which were cultured from triazole-resistant invasive aspergillosis cases. Triazole resistance variation and mixed *A fumigatus* genotypes represent a major challenge in clinical management of *Aspergillus* diseases because current molecular diagnostic tools will increasingly fail to predict the resistance phenotype, underscoring the need for improved detection methods.**

Funding **National Key Research and Development Program of China, National Natural Science Foundation of China, and Wellcome Trust.**

Copyright **© 2025 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).**

Lancet Microbe 2025; 6: 101114

Published Online July 9, 2025
https://doi.org/10.1016/j.lanmic.2025.101114

Department of Dermatology and Venerology, Peking University First Hospital, Beijing, China (Prof Y Song MD, Prof R Li MD); **Department of Medical Microbiology and Radboudumc–CWZ Center of Expertise for Mycology, Radboud University Medical Center, Nijmegen, Netherlands** (Prof Y Song, J B Buil MD, J Rhodes PhD, J Zoll PhD, M Tehupeiory-Kooreman MSc, M Ergün MD, W J G Melchers PhD, Prof P E Verweij MD); **National Clinical Research Center for Skin and Immune Diseases, Beijing, China** (Prof Y Song, Prof R Li); **Research Center for Medical Mycology, Peking University, Beijing, China** (Prof Y Song, Prof R Li); **Center for Infectious Disease Research, Diagnostics and Laboratory Surveillance, National Institute for Public Health and the Environment, Bilthoven, Netherlands** (J Zhang PhD, T Bosch PhD, Prof P E Verweij)

Correspondence to:
Prof Paul E Verweij, Department of Medical Microbiology and Radboudumc–CWZ Center of Expertise for Mycology, Radboud University Medical Center, 6500 HB Nijmegen, Netherlands
paul.verweij@radboudumc.nl

Introduction

Aspergillus fumigatus can cause a broad spectrum of diseases in humans, with recently recognised groups at risk for invasive aspergillosis including people with severe influenza and COVID-19.[1,2] The triazoles itraconazole (approved in 1992), voriconazole (2002), posaconazole (2006), and isavuconazole (2015) show activity against *Aspergillus* species, and voriconazole and isavuconazole are recommended first-line therapy for patients with invasive aspergillosis.[1] The clinical use of triazoles has improved the survival of patients with invasive aspergillosis but treatment failure remains common, which might be due to refractory underlying conditions, inability to reduce immunosuppression, and disseminated *Aspergillus* disease. Furthermore, voriconazole resistance was shown to be associated with a 25% lower 90-day survival rate in patients who are culture positive for *A fumigatus* invasive aspergillosis compared with patients with voriconazole-susceptible invasive aspergillosis.[3]

Increasingly, there are reports on triazole-resistant *A fumigatus* isolates recovered from the environment.[4] In addition to environmental hotspots that are associated with agricultural fungicide applications,[5] triazole-resistant *A fumigatus* strains were also recovered from the home environment.[6,7] A 2022 citizen science study showed that

Research in context

Evidence before this study

Previous studies have identified signature *cyp51A* gene mutations (with a tandem repeat [TR] of 34 bases [TR_{34}/Leu98His] and 46 bases [TR_{46}/Tyr121Phe/Thr289Ala]) as dominant mechanisms in triazole-resistant *Aspergillus fumigatus* isolates. We reviewed the evidence between database inception and Jan 1, 2024, for the occurrence and frequency of variations in these signature resistance mutations by searching the National Library of Medicine article database and medRxiv for preprint publications, using the terms "*Aspergillus fumigatus*", "azole", "voriconazole", "itraconazole", "posaconazole", "isavuconazole", "resistance", and "*Cyp51A*-gene". We did not apply any language restrictions to this search. We found 186 studies that reported variations of the two signature resistance mutations involving additional single-nucleotide polymorphisms (SNPs) or variations in the TR sequence or copy number. Studies since 2021 involving environmental sampling reported many *cyp51A* gene variants, but to our knowledge, there are no studies that have investigated the epidemiology of variants in triazole-resistant *A fumigatus* recovered from patient samples.

Added value of this study

This study investigated a large collection of clinical *A fumigatus* isolates, to determine variations in phenotypes and genotypes associated with cyp51A mutations as well as the clinical implications thereof. Our study showed that phenotype variations occurred in isolates with TR_{34}/Leu98His and TR_{46}/Tyr121Phe/Thr289Ala mutations, and additional SNPs in the *cyp51A* gene might confer phenotype variations, but frequently not in all isolates harbored these additional SNPs. These observations underscore the complexity of triazole resistance mechanisms in *A fumigatus*, pointing towards yet unknown non-cyp51A-mediated mechanisms that contribute to the triazole phenotype. We furthermore showed that variant isolates were involved in azole-resistant invasive aspergillosis and that pulmonary invasive aspergillosis is in principle caused by mixed genotypes. Although the clinical relevance of mixed genotype infection is limited in triazole-susceptible infection, it challenges our ability to demonstrate or rule-out resistance in triazole-resistant disease. Indeed, resistance can easily be overlooked when single *A fumigatus* colonies are used for minimal inhibitory concentration testing, which is recommended in protocols for in-vitro susceptibility testing.

Implications of all the available evidence

Resistance phenotype and genotype variations might represent the next challenge in triazole resistance management in *A fumigatus*, because we recovered resistance variants from patients with invasive aspergillosis and observed frequent mixed genotype infections. The high frequency of novel drug-resistant polymorphism combinations might result from the exceptional crossover rate that was observed in *A fumigatus*. These variations challenge our ability to reliably predict the triazole phenotype in patients with *Aspergillus* diseases. In culture-negative patients we rely on PCR, which uses specific pre-selected markers, to detect resistant *Aspergillus*. New diagnostic techniques, such as sequence-based approaches, are needed that enable detection of genotype variations, including mechanisms outside the *cyp51A* gene. Our findings furthermore underscore the need for international One Health resistance surveillance programmes for *A fumigatus*.

14% of *A fumigatus* isolates cultured from residential garden samples in the UK had non-wildtype tebuconazole minimal inhibitory concentrations (MICs).[8] Furthermore, genomic epidemiological studies showed closely related environmental and clinical triazole-resistant *A fumigatus* isolates, supporting an environment-to-person transmission route.[9]

The dominant genotypes in triazole-resistant *A fumigatus* isolates recovered from clinical and environmental samples contain mutations in the *cyp51A* gene and a tandem repeat (TR) of 34 bases (TR_{34}/Leu98His) and 46 bases (TR_{46}/Tyr121Phe/Thr289Ala) in the promoter region. However, variants that involve additional single-nucleotide polymorphisms (SNPs) or TRs in the *cyp51A* promoter region have also been reported. A TR_{34}/Leu98His/Ser297Thr/Phe495Ile variant was recovered from a clinical sample obtained in 1998,[10] indicating that TR variations were present when the TR_{34}/Leu98His mutation was first reported. Since then, this variant has been detected in various countries, and the Phe495Ile SNP was shown to be associated with resistance to imidazoles.[11,12] The 2022 citizen science study in the UK reported 17 TR_{34} variants, including TR_{34}, TR_{34}/Leu98His with various additional SNPs in the *cyp51A* gene, and one isolate with a TR_{34} duplication in the gene promoter.[8] These observations indicate that over time the number of TR variants might be increasing, although advances in molecular techniques might have also contributed to this perceived increase.

We aimed to determine the presence of variants in a large collection of clinical *A fumigatus* isolates from the Netherlands and to investigate if the number of genotype and phenotype variants increased over time and if these variants were involved in triazole-resistant invasive aspergillosis.

Methods

Study design and samples

In this genomic and phenotypic study, we screened clinical *A fumigatus* isolates from Dutch hospitals. The Radboud University Medical Centre (RUMC), Nijmegen, Netherlands, serves as mycology reference laboratory for Dutch hospitals and has collected and stored clinical *A fumigatus* isolates since Jan 6, 1994, including isolates cultured from patients admitted to the RUMC, isolates sent from hospitals for identification and in vitro susceptibility testing, and isolates sent by centres that contribute to the national *Aspergillus* resistance surveillance programme.

We included all *A fumigatus* isolates collected between Jan 6, 1994, and Dec 31, 2022. Isolates were excluded if they were from a non-clinical origin. Specimens were incubated at 28°C and 37°C and if the colony morphology was consistent with *A fumigatus*, the isolate was incubated at 48°C. At 48°C, growth of cryptic species is unlikely, but if there was concern regarding atypical colony morphology and microscopic morphology or growth rate a β-tubulin sequence was performed to identify or rule out cryptic species. The surveillance protocol was submitted to the institutional Medical Research Ethics Committee of RUMC who indicated that the study was waived from the evaluation procedure as anonymous information was used (Jan 4, 2021).

Triazole resistance screening and variant identification

When available, up to five *A fumigatus* colonies were plated on azole-containing screening agar, initially Sabouraud agar containing 4 mg/L of itraconazole and then VIPcheck screening agar (MediaProducts, Groningen, Netherlands), which contains itraconazole, voriconazole, and posaconazole. Isolates were stored at –70°C in 10% glycerol and in-vitro susceptibility testing was performed using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) microdilution reference method.[13] MICs were determined for itraconazole, voriconazole, posaconazole, isavuconazole (since 2014), and olorofim (since 2018). The following triazole resistance breakpoints were used: MIC more than 0·25 mg/L for posaconazole, more than 1 mg/L for itraconazole and voriconazole, and more than 2 mg/L for isavuconazole.[14] For *A fumigatus* isolates with a triazole-resistant phenotype, the full *cyp51A* gene including its promoter was analysed by PCR amplification and sequencing.[15] The *cyp51A* sequence (GenBank accession AF338659) was used for SNP analysis.

To investigate phenotype variation, we defined a core MIC range that included the most prevalent triazole MIC with or without two dilution steps for isolates harbouring TR_{34}/Leu98His sensu stricto or TR_{46}/Tyr121Phe/Thr289Ala sensu stricto mutations. A phenotype variant was defined as a TR_{34}/Leu98His sensu stricto or TR_{46}/Tyr121Phe/Thr289Ala sensu stricto isolate with a non-core MIC for any of the triazoles. A genotype variant was defined as an isolate that harboured additional mutations in the *cyp51A* gene or its promoter region in TR_{34}/Leu98His and TR_{46}/Tyr121Phe/Thr289Ala harbouring isolates. For Phe46Tyr harbouring isolates, promoter–335T→C/promoter–70C→T/Phe46Tyr/intron7C→T/intron66C→T/Met172Val/Glu427Lys was considered the sensu stricto genotype because these mutations were present in all genotypes. Single and multiple SNPs in the *cyp51A* gene in the absence of TRs were counted as individual variants.

Genomic and phylogenetic analysis

Genome DNA extraction was followed by the cetyltrimethylammonium bromide protocol adapted from the RUMC mycology laboratory.[16] Genomic DNA libraries were constructed with NovoSeq NGS DNA Library Prep Set (Novogene, Cambridge, UK). Prepared whole-genome libraries were sequencing on an Illumina NovaSeq 6000 at Novogene (Cambridge, UK), generating 150 bp paired-end reads. Raw reads were aligned to the *A fumigatus* reference genome Af293 (ASM265v1 GCF_000002655.1) using BWA mem v0.7.17.[17] SAMtools v1.16.1 was used to post-process the alignment prior to variant calling with GATK HaplotypeCaller v4.2.6.1 to generate genome variant call format files excluding repetitive regions (identified by RepeatMasker v4.0.6).[18] Low-confidence variants were filtered if they met at least one of the criteria: quality by depth less than 2·0, Fisher strand more than 60·0, mapping quality less than 40·0, mapping quality rank sum less than –12·5, read position rank sum less than –8·0, strand odds ratio more than 4·0, depth of coverage less than 5, and genotype quality less than 50. Filtered SNPs were mapped to genes using snpEff v5.1.

For more on **RepeatMasker** https://github.com/Dfam-consortium/RepeatMasker

For more on **snpEff** see http://snpeff.sourceforge.net/

Phylogenetic analysis was carried out on whole genome SNP data were converted to presence or absence of an SNP that has been determined as high confidence in the filtering step with respect to the reference. Any SNPs identified as low confidence in the variant filtration step were assigned to missing. These data were converted to FASTA format and maximum parsimony phylogenies were created using RAxML v8.2.9, with the general time reversible rate heterogeneity (GTRCAT) model. Maximum-likelihood phylogenies were also constructed using RAxML v8.2.9 using rapid bootstrap analysis over 750 replicates and GTRCAT model of rate heterogeneity. Final phylogenies were visualised in R v4.2.1 using the ggtree package v3.6.2.

For more on **RAxML** see https://cme.h-its.org/exelixis/web/software/raxml/

To identify important loci, we used treeWAS (a phylogenetic method for microbial genome-wide association studies) on isolates from four groups: TR_{34}/Leu98His sensu stricto with MICs within the core MIC (19 isolates) and outside the core MIC (20 isolates), and TR_{34}/Leu98His genotypic variants with MICs inside the core MIC (two variants, 30 isolates) and with MICs outside the core MIC (three variants, 13 isolates; appendix pp 3–10).[19] All raw reads were deposited to the European Nucleotide Archive (accession PRJEB74938).

See **Online** for appendix

Genetic similarity and population allocation was investigated via principal component analysis (PCA) using whole-genome SNP data in R v4.3.2 using package ade4 v1.7-22.[20] Plots were constructed in R v4.3.2 using package mapmixture v1.1.0.

For more on **mapmixture** see https://github.com/Tom-Jenkins/mapmixture

Clinical implications of triazole resistance

Clinical information such as age, underlying disease, diagnosis, therapy, and outcomes was collected for patients who had isolates cultured between Jan 1, 2017, and Dec 31, 2022, at RUMC using microbiological records with a 1-year follow-up. The initial selection criterion was a positive *A fumigatus* culture from bronchoalveolar lavage, bronchial washings, and tissue biopsies. Subsequently, the presence of invasive aspergillosis was determined using the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium and invasive fungal diseases in adult patients in intensive



Figure 1: **Number of cyp51A genotype variants in 1979 clinical triazole-resistant *Aspergillus fumigatus* isolates cultured between Jan 6, 1994, and Dec 31, 2022**

(A) Plot showing the number of isolates for each genotype over the study period. Colours indicate the various genetic backgrounds. Blue represents TR_{34}, red represents TR_{46}, orange represents TR_{53}, green represents Phe46Tyr, and light blue represents SNPs. (B) Line graph showing the mutations over time for each overall genotype category (TR_{34}, TR_{46}, TR_{53}, Phe46Tyr, and SNPs). i=intron. p=promoter region. SNP=single-nucleotide polymorphism. TR=tandem repeat.

care unit (FUNDICU) case definitions.[21,22] Data collected for invasive aspergillosis patients included underlying disease, initial and final invasive aspergillosis classification, initial and follow-up antifungal therapy, intensive care unit (ICU) admission at diagnosis, survival, triazole phenotype and genotype of all positive *A fumigatus* cultures obtained during the infection episode, and the presence of mixed *A fumigatus* infection. Mixed infection was defined as the recovery of multiple *A fumigatus* genotypes and phenotypes during the infection episode. Patients with invasive aspergillosis were excluded if other moulds were identified in bronchoalveolar lavage, bronchial washing, and tissue specimens. Patients with triazole-susceptible invasive aspergillosis were excluded if any history of a triazole-resistant isolate was recorded.

Statistical analysis

We calculated geometric mean MICs with GraphPad Prism v8.02. For calculations, we recoded MICs of more than 16 mg/L as 32 mg/L. Differences in MIC distributions were analysed using Kruskal–Wallis test, and we used the Fisher exact test to detect differences in classification according to clinical breakpoints. We used two-way ANOVA and linear regression to investigate the difference between the resistant and non-resistant isolates over time. We used a p value cutoff value of 0·01 for all three tests of association (subsequent, simultaneous, and terminal) between the triazole phenotype and genotype groups. We also performed Bonferonni p value correction. Among the three tests, the subsequent test was found to be the most effective at detecting subtle patterns of association. Therefore, we used the results from the subsequent test after applying the Bonferroni p value correction. We assessed over-represented gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms using FungiDB.[23] Triazole-resistant and triazole-susceptible invasive aspergillosis groups were compared for number of switches in antifungal therapy using the Mann–Whitney test and for survival using Kaplan–Meier estimates.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between Jan 6, 1994, and Dec 31, 2022, 12 679 *A fumigatus* isolates from clinical samples were screened for triazole resistance and the presence of cyp51A-mediated mutations (appendix p 1). Overall, 1979 (15·6%) triazole-resistant *A fumigatus* isolates were identified that harboured mutations in the *cyp51A* gene, involving five groups: TR_{34} (1432 isolates), TR_{46} (455 isolates), TR_{53} (one isolate), Phe46Tyr (38 isolates), and single SNPs (52 isolates), and a single isolate harbouring promoter–70C→T/Gly54Glu/intron7C→T/intron66C→T/Met172Val (figure 1A, B). The first resistance mutation was detected in 1995 in an isolate harbouring promoter–335T→C/promoter–70C→T/Phe46Tyr/intron7C→T/intron66C→T/Met172Val/Glu427Lys (figure 1A) and in 1998 two isolates harbouring TR_{34}/Leu98His/Ser297Thr/Phe495Ile. TR_{34}/Leu98His sensu stricto was first detected in 2000 and was since then recovered each year. The TR_{34}/Leu98His sensu stricto mutation was the most frequent resistant mechanism among the triazole-resistant isolates (1338 [67·6%] isolates) followed by TR_{46}/Tyr121Phe/Thr289Ala sensu stricto, which was present in 332 (16·8%) isolates and first observed in 2009 (table 1). The third most frequent resistance mutation was Phe46Tyr present in 38 (1·9%) isolates and first cultured in 1995, and single SNPs were first recovered in 2002 (figure 1A). An increasing trend of triazole-resistant *A fumigatus* isolates was noted, which significantly correlated with increasing number of isolates over time (appendix p 1).

According to EUCAST clinical breakpoints, a pan-triazole-resistant phenotype was present in 916 (68·5%) of 1338 TR_{34}/Leu98His sensu stricto isolates, in 106 (31·9%) of 332 TR_{46}/Tyr121Phe/Thr289Ala sensu stricto isolates, and in 16 (42·1%) of 38 Phe46Tyr isolates. Overall, 1920 (97·0%) of 1979 triazole-resistant *A fumigatus* isolates were voriconazole resistant, including 308 (15·6%) isolates with a voriconazole MIC of 2 mg/L (area of technical uncertainty resistant). Underlying resistance *cyp51A* mutations detected in the 1920 voriconazole-resistant isolates included TR_{34} (1406 isolates; 73·2%), and TR_{46} (453 isolates; 23·6%). All voriconazole-resistant isolates were cross-resistant to isavuconazole, with exception of six isolates that were isavuconazole-susceptible (MIC 1 mg/L), including four TR_{34} isolates and one isolate with promoter70C→T/Gly54Glu/intron7C→T/intron66C→T/Met172Val and promoter335T→C/promoter–70C→T/Phe46Tyr/intron7C→T/intron66C→T/Met172Val/Glu427Lys.

	Itraconazole	Voriconazole	Posaconazole	Isavuconazole*
TR_{34}/Leu98His sensu stricto (n=1338)				
Geometric mean MIC, mg/L	28·24	4·91	0·69	7·81
MIC_{50}, mg/L	32	4	0·5	8
MIC_{90}, mg/L	32	16	2	16
Range, mg/L	0·12 to >16	0·5 to >16	0·016 to >16	0·5 to >16
Resistant, % (n)	99·5% (1331)	99·0% (1324)	87·7% (1173)	97·0% (906)
ATU-resistant, % (n)	1·5% (20)	17·9% (239)	11·7% (156)	1·5% (20)
ATU-susceptible, % (n)	0	0	0	0·2% (2)
TR_{46}/Tyr121Phe/Thr289Ala sensu stricto (n=332)				
Geometric mean MIC, mg/L	5·95	26·68	0·62	24·45
MIC_{50}, mg/L	8	32	0·5	32
MIC_{90}, mg/L	32	32	1	32
Range, mg/L	0·25 to >16	1 to >16	0·125 to >16	2 to >16
Resistant, % (n)	71·7% (238)	99·7% (331)	80·1% (266)	99·7% (1)
ATU-resistant, % (n)	14·5% (48)	0	3·6% (12)	0·3% (1)
ATU-susceptible, % (n)	0	0	14·8% (49)	0

ATU=area of technical uncertainty. MIC=minimum inhibitory concentration. MIC_{50}=minimum inhibitory concentration that inhibits the growth of 50% of the tested microorganisms. MIC_{90}=minimum inhibitory concentration that inhibits the growth of 90% of the tested microorganisms. TR=tandem repeat. *Isavuconazole MICs were not measured before 2014.

Table 1: **Triazole phenotypes for TR_{34}/Leu98His sensu stricto and TR_{46}/Tyr121Phe/Thr289Ala sensu stricto isolates**

For more on **GraphPad Prism** see https://www.graphpad.com

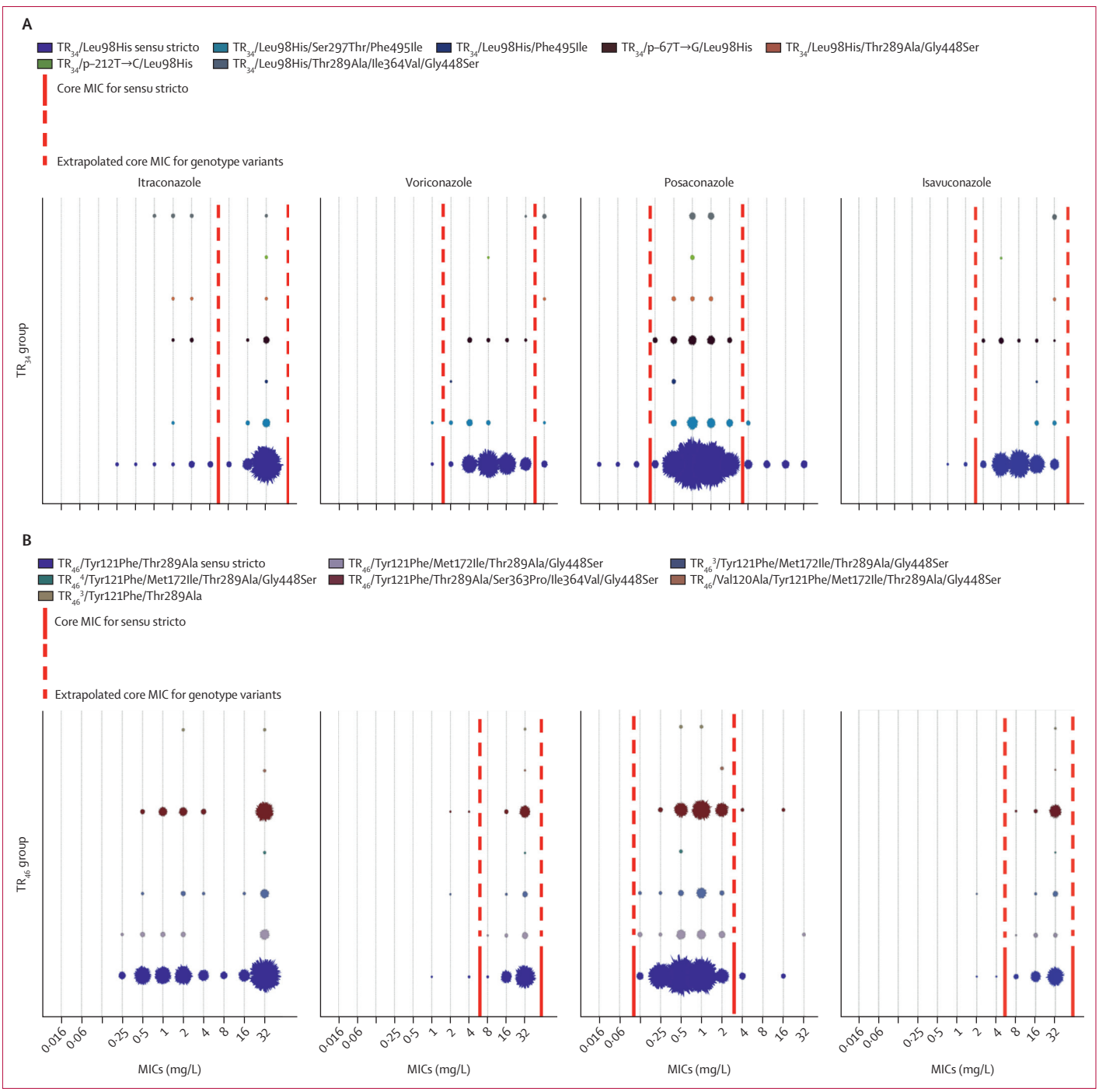


Figure 2: **Triazole phenotypes of TR_{34}/Leu98His sensu stricto isolates and TR_{34} genotype variants (A), and TR_{46}/Tyr121Phe/Thr289Ala sensu stricto isolates TR_{46} genotype variants (B)**
The size of the dots corresponds with the proportion of isolates with that specific MIC. The red lines indicate the core MIC values. TR_{34}/Leu98His sensu stricto and TR_{46}/Tyr121Phe/Thr289Ala sensu stricto isolates with MICs outside the range shown in the figure were considered non-core phenotype variants. cyp51A-genotype variants are indicated in other colours. For these isolates the core MIC values were derived from the TR_{34}/Leu98His sensu stricto and TR_{46}/Tyr121Phe/Thr289Ala sensu stricto, indicated with the red dotted lines. MIC=minimal inhibitory concentration. p=promoter region. TR=tandem repeat.

A broad range of MICs was observed for TR_{34}/Leu98His sensu stricto and TR_{46}/Tyr121Phe/Thr289Ala sensu stricto for all triazoles (figure 2). For each triazole, a core MIC range could be defined except for itraconazole in TR_{46}/Tyr121Phe/Thr289Ala sensu stricto isolates, where the MIC distribution indicated the presence of two populations (figure 2). Among the 1338 TR_{34}/Leu98His sensu stricto isolates 99 (7·4%) exhibited an MIC outside the core

	Isolation date	Phenotype			
		Itraconazole, mg/L	Voriconazole, mg/L	Posaconazole, mg/L	Isavuconazole, mg/L
TR_{34}/Leu98His sensu stricto geometric mean MIC	..	28·24	4·91	0·69	7·81
Core MIC range	..	8 to >16	1 to 16	0·12 to 2	2 to >16
TR_{46}/Tyr121Phe/Thr289Ala sensu stricto geometric mean MIC	..	5·95	26·68	0·62	24·45
Core MIC range	..	..	8 to >16	0·12 to 2	8 to >16
TR_{34}/Leu98His/Thr289Ala/Ile364Val/Gly448Ser (10 [100%] of 10 isolates)					
Strain: V309-77	Jan 31, 2020	1*	>16*	1	>16
Strain: M010-39	Nov 04, 2020	2*	>16*	1	>16
Strain: M027-69	May 27, 2021	0·5*	>16*	0·5	>16
Strain: M029-04	June 08, 2021	2*	>16*	1	>16
Strain: M033-22	Aug 09, 2021	1*	>16*	1	>16
Strain: M037-21	Sept 29, 2021	1*	>16*	0·5	>16
Strain: M046-26	Dec 30, 2021	0·5*	>16*	1	>16
Strain: M061-48	Aug 04, 2022	2*	>16*	0·5	>16
Strain: M061-58	Aug 05, 2022	1*	16	0·5	>16
Strain: M062-29	Aug 16, 2022	1*	>16*	0·5	>16
TR_{34}/Leu98His/Thr289Ala/Gly448Ser (3 [100%] of 3 isolates)					
Strain: v165-63	June 27, 2014	1*	>16*	1	>16
Strain: v253-11	May 18, 2018	1*	>16*	0·25	>16
Strain: M072-67	Dec 15, 2022	2*	>16*	0·5	>16
TR_{34}/promoter-67T→G/Leu98His (6 [18·8%] of 32 isolates)					
Strain: v259-28	July 26, 2018	2*	2	0·5	2
Strain: v269-74	Nov 29, 2018	2*	2	0·25	4
Strain: v272-27	Dec 06, 2018	2*	2	0·25	2
Strain: v272-32	Dec 06, 2018	2*	2	0·5	2
Strain: v306-29	Dec 12, 2019	2*	2	0·5	4
Strain: M031-27	July 09, 2021	1*	2	0·25	2
TR_{34}/Leu98His/Ser297Thr/Phe495Ile (5 [10·6%] of 47 isolates)					
Strain: v085-79	Aug 23, 2009	>16	>16*	0·5	>16
Strain: v141-26	Oct 04, 2012	1*	>16*	0·5	>16
Strain: v206-01	Aug 10, 2016	>16	2	4*	>16
Strain: V255-19	May 31, 2018	>16	0·5*	0·5	16
Strain: M042-45	Nov 29, 2021	1*	>16*	0·5	>16
TR_{46}/Tyr121Phe/Thr289Ala/Ser363Pro/Ile364Val/Gly448Ser (4 [4·1%] of 78 isolates)					
Strain: v255-56	May 30, 2018	>16	>16	16*	>16
Strain: M005-25	Sept 04, 2020	>16	>16	4*	>16
Strain: M045-14	Dec 21, 2021	>16	4*	0·25	8
Strain: M052-47	April 05, 2022	>16	4*	0·25	8

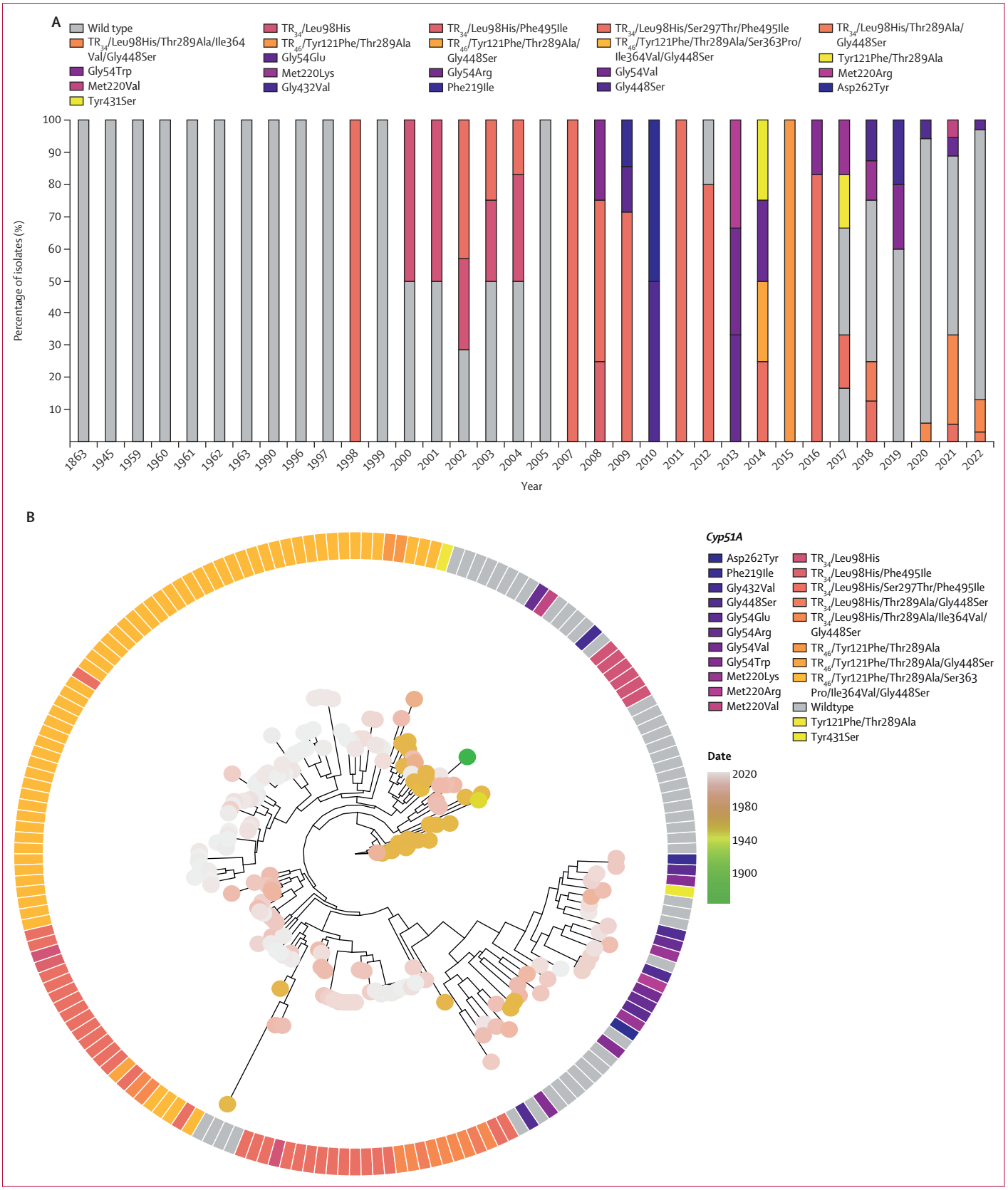
MIC=minimum inhibitory concentration. TR=tandem repeat. *MICs outside of the core MIC distribution.

Table 2: **TR_{34} and TR_{46} genotype variants with triazole phenotypes that are not within the core MIC distribution of TR_{34}/Leu98His sensu stricto and TR_{46}/Tyr121Phe/Thr289Ala sensu stricto isolates**

MIC (appendix pp 11–15). Itraconazole non-core phenotype variants (36 isolates) showed MICs of less than 4 mg/L, and for voriconazole most variant isolates (15 [62·5%] of 24) showed MICs of more than 16 mg/L (appendix pp 11–15). Among the 332 TR_{46}/Tyr121Phe/Thr289Ala sensu stricto isolates, nine (2·7%) exhibited an MIC outside the defined core MIC (appendix pp 11–15).

In the TR_{34} genetic background, six (19·4%) of the 31 genotype variants were observed, involving 94 (6·6%) isolates (figure 1A). In the TR_{46} genetic background, six variants (19·4%) of the 31 were found, involving 123 (27·0%) isolates. Unlike TR_{34} isolates, variations in TR_{46} duplication number were observed, including 17 (3·7%) isolates with three TRs (TR_{46}^{3}/Tyr121Phe/Met172Ile/Thr289Ala/Gly448Ser) and one isolate with four TRs, together with two SNPs in the *cyp51A* gene: TR_{46}^{4}/Tyr121Phe/Met172Ile/Thr289Ala/Gly448Ser (figure 1A).

Of the 38 isolates with the Phe46Tyr genetic background, 14 isolates harboured additional Asn248Thr/Asp255Glu SNPs and one isolate harboured Met220Ile (figure 1A).



(Figure 3 continues on next page)

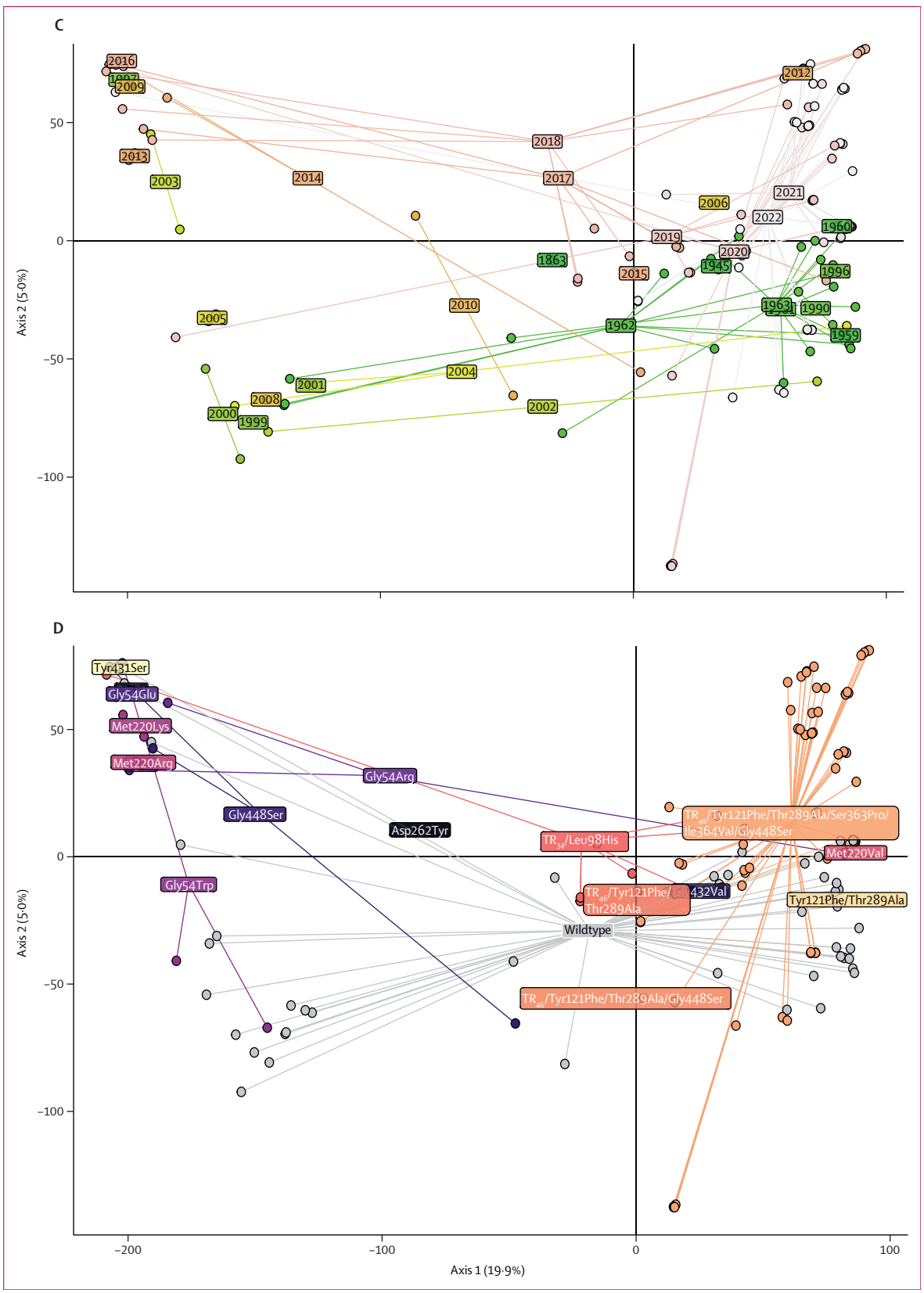


***Figure 3*: Whole-genome sequencing of 181 triazole-susceptible and triazole-resistant *Aspergillus fumigatus* isolates**
(A) Normalised bar plot of percentage of 181 isolates along time according to *cyp51A* polymorphism. (B) Maximum parsimony phylogeny and *cyp51A* phenotypic variation of 181 isolates. (C) Principal component analysis of the year of isolation. The first two principal components show no distinction on time. (D) Principal component analysis based on *cyp51a* genotype shows TR_{34} and TR_{46} isolates are drawn from different backgrounds, with some overlap. Scale bar represents an approximate number of single nucleotide polymorphisms. TR=tandem repeat.

	Single-nucleotide polymorphism type	Gene	Description
Chromosome 1			
Position: 4 707 904	Upstream	AFUA_1G17210	Protein of unknown function; orthologs are F-box containing proteins
Position: 4 707 979	Upstream	AFUA_1G17210	Protein of unknown function; orthologs are F-box containing proteins
Position: 4 708 022	Upstream	AFUA_1G17210	Protein of unknown function; orthologs are F-box containing proteins
Chromosome 2			
Position: 2 050 524	Missense (Arg355Cys)	AFUA_2G08000	Orthologs are transmembrane proteins
Position: 2 050 607	Missense (Gly327Glu)	AFUA_2G08000	Orthologs are transmembrane proteins
Position: 2 052 509	Upstream	AFUA_2G08000	Orthologs are transmembrane proteins

*The four groups are TR_{34}/Leu98His sensu stricto with MICs within the core MIC (19 isolates) and outside the core MIC (20 isolates), and TR_{34}/Leu98His genotypic variants with MICs inside the core MIC (two variants, 30 isolates) and with MICs outside the core MIC (three variants, 13 isolates).

Table 3*: Significant loci identified by treeWAS as associated with the four groups

14 (45·2%) of the 31 cyp51A variant SNPs were observed in 52 (2·6%) of 1979 triazole-resistant *A fumigatus* isolates (figure 1A).

Gly448Ser was relatively common and present in various genetic backgrounds, including TR_{46} (five variants) and TR_{34} (two variants; appendix p 1). In addition to Gly448Ser, both TR_{34} variants also harboured Thr289Ala, a substitution that is characteristic of the TR_{46} genotype. Mutations in the gene promoter region were also frequent, with the 67T→G substitution most often found in isolates harbouring TR_{34}/Leu98His (32 isolates; appendix p1).

Five cyp51A-variant genotypes (28 isolates) in TR_{34} and TR_{46} isolates conferred triazole phenotypes outside the TR_{34}/Leu98His sensu stricto and TR_{46}/Tyr121Phe/Thr289Ala sensu stricto core MIC distribution (figure 2; table 2). In TR_{34}-genotype variants, low itraconazole MICs (range 0·5–2 mg/L) were the most prevalent non-core phenotype (21 [87·5%] of 24 isolates), commonly combined with high voriconazole MICs (MIC >16 mg/L, 15 [62·5%] of 24 isolates; table 2). In TR_{46}-genotype variants, two isolates showed low voriconazole MICs, and two isolates high posaconazole MICs (table 2). In two TR_{34}-genotype variants (TR_{34}/Leu98His/Thr289Ala/Ile364Val/Gly448Ser and TR_{34}/Leu98His/Thr289Ala/Gly448Ser) a non-core phenotype was observed in all isolates, and in the remaining variants only a minority of isolates exhibited a non-core phenotype (table 2).

Overall, 325 (17·2%) of 1887 TR-isolates showed triazole phenotype or cyp51A-genotype variation, or both. The cyp51A genotype and phenotype variants increased over time and correlated with increasing TR-isolate numbers (appendix p 2).

We performed whole-genome sequencing on 213 *A fumigatus* isolates (appendix pp 3–10), of which 47 contained no polymorphisms in the coding or promoter regions of *cyp51A* and were classified as wildtype (figure 3A). The remaining 166 isolates displayed either polymorphisms in the coding or promoter regions of *cyp51A*, or both (appendix pp 3–10). Phylogenetic analysis revealed that most of the wildtype isolates clustered together, and TR_{34} and TR_{46} isolates also clustered together (figure 3B). Although there was no distinction observed based on PCA of the date of isolation (figure 3C), confirming the observation in the phylogenetic analysis, there was some distinction along the second PCA observed between isolates with combinations of TR_{34}-based and TR_{46}-based polymorphisms (figure 3D); some overlap between these two groups was observed.

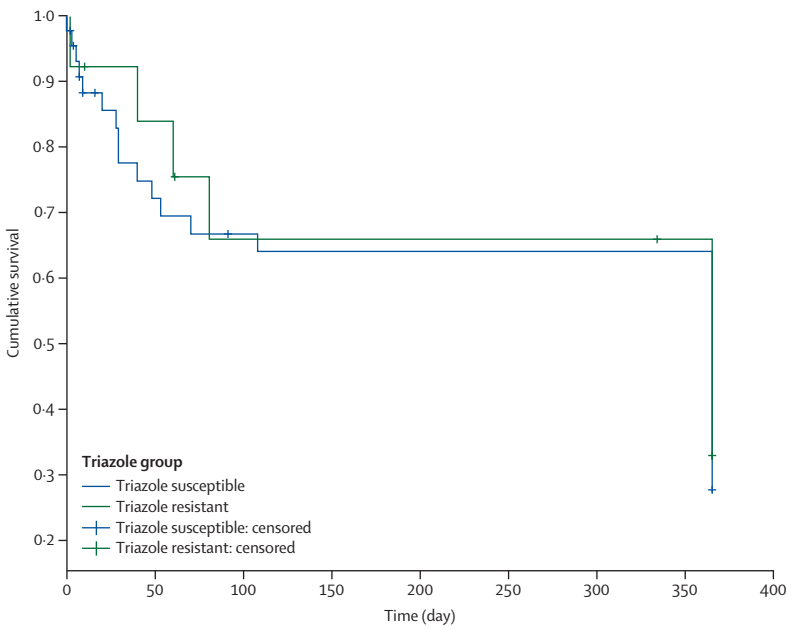
TreeWAS identified 552 significant loci using the subsequent test as associated non-core MICs in TR_{34}/Leu98His variant isolates. No significant loci were found associated with core or non-core MIC phenotypes in TR_{34}/Leu98His sensu stricto isolates. These 552 loci mapped to 109 genes (appendix pp 16–70), which were over-represented for KEGG pathway terms chlorocyclohexane and chlorobenzane degradation (p=0·032) and diterpenoid biosynthesis (p=0·045; table 3); these are components of azoles, suggesting that some pathways are more upregulated in isolates with additional *cyp51a* polymorphisms. Additionally, gene ontology terms associated with recombination were also over-represented in these 109 genes, suggesting recombination is playing a crucial role in determining core versus non-core triazole phenotype in TR_{34}/Leu98His variant isolates.

Between Jan 1, 2017 and Dec 31, 2022, 2010 cultures yielded *A fumigatus* obtained from 1189 patients. Most cultures were from sputum (n=1296) and bronchial aspirates (n=258), and 210 patients had positive cultures from bronchoalveolar lavage, bronchial washings, and tissue. Invasive aspergillosis could be classified in 59 patients, including 17 proven and 42 probable cases. Although the FUNDICU case definition requires a CT scan to classify probable invasive pulmonary aspergillosis in patients who are non-neutropenic, this criterion was omitted because many ventilated ICU patients do not undergo CT imaging. 13 (22·0%) of the 59 patients had triazole-resistant invasive aspergillosis (table 4). Compared with 46 (78·0%) of 59 patients with triazole-susceptible invasive aspergillosis (appendix pp 71–74), the proportion of ICU patients was similar for both groups (nine [69·2%] of 13 for triazole-resistant invasive aspergillosis and 32 [69·6%] of 46 for triazole-susceptible invasive aspergillosis), as was the overall mortality (seven [53·8%] of 13 for triazole-resistant invasive aspergillosis and 23 [50·0%] of 46 for triazole-susceptible invasive aspergillosis; figure 4). TR-mediated resistance genotypes were involved in 12 (92·3%) of 13 patients with triazole-resistant invasive aspergillosis, including three genotype variants (table 4). Furthermore, mixed infection was observed in 11 (84·6%) of 13 patients, including four patients with multiple resistance genotypes. The two patients without mixed infection were diagnosed through biopsy of a single lesion (table 4). In both patient groups, most patients received initial antifungal therapy that covered triazole resistance (ie, triazole and echinocandin or liposomal amphotericin, or liposomal amphotericin monotherapy), which is recommended by the Dutch

	Year	Age, years	Underlying disease	Initial classification	Final classification	ICU	Initial therapy	Follow-up therapy	Outcome	Mixed infection	Resistance genotype	Resistance phenotype					
												AmB, mg/L	ITZ, mg/L	VCZ, mg/L	POS, mg/L	ISA, mg/L	OLO, mg/L
Patient 1	2017	67	Oropharynx carcinoma	Probable IPA	Probable IPA	Yes	VCZ	VCZ and AFG; VCZ	Died 60 days after therapy	S; R	TR_{34}/Leu98His	0·5	16	2	0·5	4	..
Patient 2	2017	73	CLL (ibrutinib), COPD, and NTM	Probable IPA	Probable IPA	Yes	VCZ and AFG	AFG; L-AmB and AFG; VCZ and AFG	Died 40 days after therapy	S; R; R	WT; TR_{34}/Leu98His	0·5; 0·5	>16; >16	4; 2	0·5; 0·5	4; 4	0·031; <0·016
Patient 3	2017	58	Legionella pneumonia	Unclassified	Proven IPA	Yes	VCZ	None	Died 2 days after therapy	S; R; R	WT; TR_{46}/Tyr121Phe/Thr289Ala	0·25; 0·5	>16; 2	2; >16	0·5; 1	4; >16	0·031; 0·063
Patient 4	2018	55	Rheumatoid arthritis and COPD	Probable IAPA	Probable IAPA		VCZ and CAS	VCZ; VCZ and CAS; VCZ, L-AmB, and nL-AmB; VCZ and nL-AmB	Died 10 days after therapy	S; R	TR_{34}/Leu98His	0·5	>16	2	0·5	8	0·016
Patient 5	2019	38	Rheumatoid arthritis	Probable IAPA	Probable IAPA	Yes	VCZ and AFG	VCZ and L-AmB	Alive	S; R	TR_{46}/Tyr121Phe/Met172Ile/Thr289Ala/Gly448Ser	0·25	0·5	>16	0·25	>16	0·031
Patient 6	2020	62	None	Proven osteomyelitis following mastoidectomy	Proven osteomyelitis	No	L-AmB and tL-AmB	AFG; AFG and ISA; VCZ, AFG, and tVCZ	Alive	No	TR_{34}/Leu98His	1	>16	4	1	4	0·031
Patient 7	2020	67	Diabetes and hypertension	Probable CAPA and TBA	Probable CAPA+TBA	Yes	VCZ and AFG	VCZ, AFG, and nL-AmB; L-AmB and nL-AmB	Died 81 days after therapy	S; R	TR_{34}/Leu98His	0·5	>16	4	0·5	8	0·063
Patient 8	2021	58	Primary hyperaldosteronism	Probable CAPA	Probable CAPA	Yes	VCZ and AFG	L-AmB; POS and AFG	Alive	S; R	TR_{46}/Tyr121Phe/Thr289Ala	1	0·5	>16	0·25	>16	0·031
Patient 9	2021	61	None	Probable CAPA	Probable CAPA	Yes	VCZ and AFG	L-AmB	Alive	R; R; R	TR_{34}/promoter–67T→G/Leu98His; WT; TR_{34}/Leu98His	0·5; 0·5; 0·5	>16; >16; >16	4; 2; 4	1; 0·25; 0·5	8; 4; 8	0·063; 0·031; 0·031
Patient 10	2021	73	Diabetes	Probable CAPA	Probable CAPA	Yes	VCZ and AFG	ISA and AFG	Alive	S; R	TR_{34}/Leu98His	0·25	>16	8	1	16	0·063
Patient 11	2021	33	Kidney transplant	CAPA (unclassified)	Proven disseminated IA	Yes	POS and AFG	OLO; OLO and L-AmB	Died 334 days after therapy	R; R	TR_{46}/Tyr121Phe/Thr289Ala/Ser363Pro/Ile364Val/Gly448Ser; TR_{34}/Leu98His	0·5; 1	>16; >16	4; 8	0·25; 0·5	8; 16	0·031; 0·031
Patient 12	2022	54	AML and MUD alloSCT	Proven IPA	Proven IPA	No	VCZ	ISA; L-AmB; MFG; L-AmB; ISA and MFG	Alive	No	TR_{34}/Leu98His	1	>16	4	1	8	0·016
Patient 13	2022	31	CGD	Proven IPA	Proven disseminated IA	No	ISA and OLO	OLO and AFG; OLO, AFG, and L-AmB	Died 61 days after therapy	S; R	WT	0·5	2	2	0·25	4	0·125

AFG=anidulafungin. allSCT=allogeneic stem cell transplantation. AmB=amphotericin. AML=acute myeloid leukaemia. CAPA=COVID-19-associated pulmonary aspergillosis. CAS=caspofungin. CGD=chronic granulomatous disease. CLL=chronic lymphoblastic leukaemia. COPD=chronic obstructive pulmonary disease. IA=invasive aspergillosis. IAPA=influenza-associated pulmonary aspergillosis. ICU=intensive care unit. IPA=invasive pulmonary aspergillosis. ISA=isavuconazole. L-AmB=liposomal amphotericin B. MFG=micafungin. MUD=matched unrelated donor. nL-AmB=nebulized liposomal amphotericin B. NTM=non-tuberculous mycobacteria. OLO=olorofim. POS=posaconazole. R=triazole-resistant. S=triazole susceptible. TBA=invasive tracheobronchial aspergillosis. tL-AmB=topical liposomal amphotericin B. TR=tandem repeat. tVCZ=topical voriconazole. VCZ=voriconazole. WT=wildtype.

Table 4: **Characteristics of 13 patients with proven or probable triazole-resistant invasive aspergillosis**



Day	0	42	84	120
Triazole susceptible	45	28	25	23
Triazole resistant	13	10	7	7

Figure 4: **Kaplan–Meier survival analysis of patients with triazole-resistant invasive aspergillosis and triazole-susceptible invasive aspergillosis**
This analysis estimated the survival function from lifetime data to compare survival distributions between the two groups.

National Invasive Mycoses Guideline published in December 2017 (nine [90·0%] of ten triazole-resistant invasive aspergillosis cases since 2018 and 34 [87·2%] of 39 triazole-susceptible cases since 2018). Compared with triazole-susceptible invasive aspergillosis, significantly more antifungal treatment switches were made in patients with triazole-resistant infection (mean rank 25·98 for triazole-susceptible compared with 44·23 for triazole-resistant; p<0·0001). Olorofim showed low MICs against both triazole-susceptible and triazole-resistant *A fumigatus* isolates (table 4; appendix pp 71–74).

Discussion

Analysis of a large collection of clinical *A fumigatus* isolates collected over 29 years showed that *cyp51A* genotype and triazole phenotype variation was frequent in resistant isolates. Non-core MICs were observed both in TR_{34}/Leu98His sensu stricto and TR_{46}/Tyr121Phe/Thr289Ala sensu stricto isolates, and in *cyp51A* genotype variants. Furthermore, triazole phenotype variations might be more frequent in isolates collected since 2017 compared with those collected from 1994 to 2004, which was also observed in genomic comparisons.

Our data indicate that over time *A fumigatus* acquires mutations, some of which might alter the triazole resistance phenotype. These mutations are detectable when *cyp51A* is involved, but we hypothesise that mutations outside cyp51A are likely responsible for part of the phenotypic variation we observed. With the generation of de novo mutations in asexually reproducing *A fumigatus* and continued triazole selection pressure in the environment, our observation is not unexpected. Whole-genome sequencing showed that although isolates with combinations of TR_{34}-based and TR_{46}-based polymorphisms in *cyp51A* seem to be derived from separate populations, there is some overlap. One possible explanation is (sexual) recombination between isolates with these distinct genetic backgrounds, creating new combinations of triazole resistance polymorphisms (eg, TR_{34}/Leu98His/T289A); more combinations have been observed since 2017 during the period of this study (appendix pp 71–74). The high crossover rate in *A fumigatus*,[24] alludes to a future with more novel drug-resistant polymorphism combinations being observed. Recombination was also seen to be involved in the determination of core versus non-core MIC phenotypes in isolates included in this study; many significant loci in TR_{34}/Leu98His *cyp51A* variant isolates that were over-represented for gene ontology terms were associated with recombination, suggesting that recombination is driving phenotypic variation and potentially patient infection experience such as therapeutic outcomes and disease progression. Therefore, it is crucial to routinely perform susceptibility testing of novel resistance polymorphism combinations as part of surveillance programmes, and to assess patient outcomes.

We recovered resistance variants from patients with proven or probable invasive aspergillosis underscoring the potential of these variants to cause invasive disease. Most patients were admitted to the ICU, which might be due to culture positivity as selection criterion. In patients with haematological malignancy and neutropenia, invasive aspergillosis diagnosis is frequently based on biomarkers, and positive cultures are infrequent. Mortality in triazole-resistant and triazole-susceptible invasive aspergillosis was similar, which might be due to initial antifungal therapy that covered triazole resistance as recommended in our national guideline. Although our previous cohort study showed significantly higher mortality in patients with voriconazole-resistant invasive aspergillosis compared with patients with voriconazole-susceptible invasive aspergillosis, the mortality in both groups was similar for patients admitted to the ICU.[3] Other factors probably have greater impact on survival in critically ill patients in addition to triazole resistance. Nevertheless, the high number of antifungal drug changes in patients with triazole-resistant invasive aspergillosis underscores the challenges faced, including the need for high triazole exposure (especially posaconazole) and continued combination therapy with associated drug toxicity and interactions.

Our study indicates that pulmonary invasive aspergillosis in principle involves multiple *A fumigatus* genotypes, because lung nodules originate from genetically distinct spores.[25] Both patients with one genotype had been biopsied

from a single lesion in lung and bone (table 4). Multiple resistance genotypes or phenotypes including resistant isolates with a wildtype cyp51A sequence as well as cyp51A genotype variants are a concern for resistance detection. *Aspergillus* resistance PCR assays use predefined markers (eg, TR_{34}, Leu98His, Tyr121Phe, and Thr289Ala) to detect resistance, but will not detect cyp51A-genotype or phenotype variants and non-cyp51A-mediated resistance. Phenotypic resistance detection through agar-based screening of multiple *A fumigatus* colonies and MIC-testing will be the most reliable method to detect MIC-variation. However, many patients are culture-negative and rely on molecular testing to detect resistance. Other techniques, such as sequence-based approaches, will be required in clinical microbiology to enable reliable detection of resistance. However, gene editing experiments will be needed to understand the role of individual SNPs in the *cyp51A* gene as well as identifying new resistance hotspots. The TR_{34}/Leu98His/Thr289Ala/Gly448Ser variant incorporates two mutations that are known to confer triazole resistance.[26] TR_{34}/Leu98His confers high itraconazole MICs and isolates with a Gly448Ser point mutation show high MICs for both voriconazole and itraconazole.[27] The Thr289Ala mutation is not associated with increased triazole MICs, but confers triazole resistance when combined with Tyr121Phe or TR_{46}, or both.[28] Therefore, each SNP will need to be investigated individually and in combination to understand the associated phenotype.

Our study has some limitations. First, the study was limited by using cultures as the denominator, which may include multiple cultures from the same patient and excludes culture-negative patients with aspergillosis, that were diagnosed using other techniques such as *Aspergillus* antigen detection or *Aspergillus* PCR. Second, there will have been variation in resistance detection methods over time. Third, multiple isolates from the same patient could impact on the resistance frequency and variation. Finally, we were unable to retrieve patient data for all isolates because some of the isolates were collected anonymously for our resistance surveillance programme. However, the data collected for the RUMC provide an indication regarding sample types and clinical implications of triazole resistance.

Our study showed phenotype or genotype variation in 17·2% of clinical triazole-resistant *A fumigatus* isolates, and involvement of these variants in triazole-resistant invasive aspergillosis. Mixed infections involving resistance variants represents the next major challenge in triazole resistance management as our current molecular diagnostic tools will increasingly fail to accurately predict the triazole phenotype. New approaches are needed to detect resistance variants in clinical specimens.

Contributors

PEV, JBB, and WJGM conceived and designed the study. YS, JR, and PEV performed the data analysis and have verified the data reported in the study. YS, JZo, MT-K, and ME conducted all experiments and performed the statistical analysis of the data. YS, PEV, JBB, JZo, JZh, RL, TB, JR, ME, and WJGM discussed the experiments and results. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication. YS, JR, and PEV wrote the draft manuscript; all authors reviewed the different drafts and approved the final version of the manuscript.

Declaration of interests

JBB received research grants from Gilead Sciences and F2G. PEV received grants for research, lectures and consultancy from Gilead Sciences, Mundipharma, Shionogi, and F2G. All other authors declare no competing interests.

Data sharing

All genotypic variants are shown in figure 1A. All data requests should be submitted to the corresponding author. Requests will be assessed for scientific rigour before being granted and a data-sharing agreement might be required.

Acknowledgments

This study was supported by National Key Research and Development Program of China (2021YFC2300400) and National Natural Science Foundation of China (82272354) supporting YS and RL. Parts of the study were supported by a grant from the Wellcome Trust (219551/Z/19/Z) to JR and PEV.

References

1 Ullmann AJ, Aguado JM, Arikan-Akdagli S, et al. Diagnosis and management of *Aspergillus* diseases: executive summary of the 2017 ESCMID-ECMM-ERS guideline. *Clin Microbiol Infect* 2018; **24** (suppl 1): e1–38.
2 Alanio A, Dellière S, Fodil S, Bretagne S, Mégarbane B. Prevalence of putative invasive pulmonary aspergillosis in critically ill patients with COVID-19. *Lancet Respir Med* 2020; **8:** e48–49.
3 Lestrade PP, Bentvelsen RG, Schauwvlieghe AFAD, et al. Voriconazole resistance and mortality in invasive aspergillosis: a multicenter retrospective cohort study. *Clin Infect Dis* 2019; **68:** 1463–71.
4 Barber AE, Riedel J, Sae-Ong T, et al. Effects of agricultural fungicide use on *Aspergillus fumigatus* abundance, antifungal susceptibility, and population structure. *MBio* 2020; **11:** e02213–20.
5 Schoustra SE, Debets AJM, Rijs AJMM, et al. Environmental hotspots for azole resistance selection of *Aspergillus fumigatus*, the Netherlands. *Emerg Infect Dis* 2019; **25:** 1347–53.
6 Lavergne RA, Chouaki T, Hagen F, et al. Home environment as a source of life-threatening azole-resistant *Aspergillus fumigatus* in immunocompromised patients. *Clin Infect Dis* 2017; **64:** 76–78.
7 Paluch M, Lejeune S, Hecquet E, Prévotat A, Deschildre A, Fréalle E. High airborne level of *Aspergillus fumigatus* and presence of azole-resistant TR_{34}/L98H isolates in the home of a cystic fibrosis patient harbouring chronic colonisation with azole-resistant H285Y *A. fumigatus*. *J Cyst Fibros* 2019; **18:** 364–67.
8 Shelton JMG, Collins R, Uzzell CB, et al. Citizen science surveillance of triazole-resistant *Aspergillus fumigatus* in United Kingdom residential garden soils. *Appl Environ Microbiol* 2022; **88:** e0206121.
9 Rhodes J, Abdolrasouli A, Dunne K, et al. Population genomics confirms acquisition of drug-resistant *Aspergillus fumigatus* infection by humans from the environment. *Nat Microbiol* 2022; **7:** 663–74.
10 Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE, Melchers WJ. Azole resistance profile of amino acid changes in *Aspergillus fumigatus* CYP51A based on protein homology modeling. *Antimicrob Agents Chemother* 2010; **54:** 2425–30.
11 Liu M, Zeng R, Zhang L, et al. Multiple cyp51A-based mechanisms identified in azole-resistant isolates of *Aspergillus fumigatus* from China. *Antimicrob Agents Chemother* 2015; **59:** 4321–25.
12 Chen Y, Li Z, Han X, et al. Elevated MIC values of imidazole drugs against *Aspergillus fumigatus* isolates with TR_{34}/L98H/S297T/F495I mutation. *Antimicrob Agents Chemother* 2018; **62:** e01549–17.
13 European Committee on Antimicrobial Susceptibility Testing. Antifungal Susceptibility Testing. https://www.eucast.org/ast_of_fungi/ (accessed Dec 4, 2020).
14 Arendrup MC, Friberg N, Mares M, et al. How to interpret MICs of antifungal compounds according to the revised clinical breakpoints v. 10.0 European committee on antimicrobial susceptibility testing (EUCAST). *Clin Microbiol Infect* 2020; **26:** 1464–72.

15 Mellado E, Diaz-Guerra TM, Cuenca-Estrella M, Rodriguez-Tudela JL. Identification of two different 14-alpha sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species. *J Clin Microbiol* 2001; **39:** 2431–38.

16 Arentshorst M, Ram AF, Meyer V. Using non-homologous end-joining-deficient strains for functional gene analyses in filamentous fungi. *Methods Mol Biol* 2012; **835:** 133–50.

17 Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA–MEM. *arXiv* 2013; published online March 16. https://doi.org/10.48550/arXiv.1303.3997 (preprint).

18 Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009; **25:** 2078–79.

19 Collins C, Didelot X. A phylogenetic method to perform genome-wide association studies in microbes that accounts for population structure and recombination. *PLOS Comput Biol* 2018; **14:** e1005958.

20 Ter Braak CJ, Peres-Neto P, Dray S. A critical issue in model-based inference for studying trait-based community assembly and a solution. *PeerJ* 2017; **5:** e2885.

21 Donnelly JP, Chen SC, Kauffman CA, et al. Revision and update of the consensus definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium. *Clin Infect Dis* 2020; **71:** 1367–76.

22 Bassetti M, Giacobbe DR, Agvald-Ohman C, et al. Invasive fungal diseases in adult patients in intensive care unit (FUNDICU): 2024 consensus definitions from ESGCIP, EFISG, ESICM, ECMM, MSGERC, ISAC, and ISHAM. *Intensive Care Med* 2024; **50:** 502–15.

23 Alvarez-Jarreta J, Amos B, Aurrecoechea C, et al. VEuPathDB: the eukaryotic pathogen, vector and host bioinformatics resource center in 2023. *Nucleic Acids Res* 2024; **52:** D808–16.

24 Auxier B, Debets AJM, Stanford FA, et al. The human fungal pathogen *Aspergillus fumigatus* can produce the highest known number of meiotic crossovers. *PLoS Biol* 2023; **21:** e3002278.

25 Kolwijck E, van der Hoeven H, de Sévaux RG, et al. Voriconazole-susceptible and voriconazole-resistant *Aspergillus fumigatus* coinfection. *Am J Respir Crit Care Med* 2016; **193:** 927–29.

26 Abdolrasouli A, Rhodes JL. Phenotypic variants of azole-resistant *Aspergillus fumigatus* that co-exist in human respiratory samples are genetically highly related. *Mycopathologia* 2022; **187:** 497–508.

27 Pelaez T, Gijón P, Bunsow E, et al. Resistance to voriconazole due to a G448S substitution in *Aspergillus fumigatus* in a patient with cerebral aspergillosis. *J Clin Microbiol* 2012; **50:** 2531e4.

28 Snelders E, Camps SM, Karawajczyk A, et al. Genotype-phenotype complexity of the TR_{46}/Y121F/T289A cyp51A azole resistance mechanism in *Aspergillus fumigatus*. *Fungal Genet Biol* 2015; **82:** 129–35.