Resistance in human pathogenic yeasts and filamentous fungi: prevalence, underlying molecular mechanisms and link to the use of antifungals in humans and the environment

Antifungal drug resistance in pathogenic fungi

Rasmus Hare Jensen

This review has been accepted as a thesis together with six previously published papers by University of Copenhagen and defended on January 29th 2016.

Tutor(s): Maiken Cavling Arendrup & Helle Krogh Johansen

Official opponents: Henrik Torkild Westh, Niels Frimodt-Møller & Jesus Guinea Ortega

Correspondence: Rasmus Hare Jensen, office 43-316, Department, Microbiology and Infection Control, Statens Serum Institut, Artillerivej 5, 2300 Copenhagen S, Denmark

E-mail: RMJ@ssi.dk, telephone: +4532683845

Dan Med J 2016;63(10)B5288

LIST OF PAPERS

This PhD thesis is based on the following original papers

(I) Jensen RH, Johansen HK, Arendrup MC. 2012. Stepwise development of homozygous S80P substitution in *FKS1*p conferring echinocandin resistance in *Candida tropicalis*. Antimicrob. Agents Chemother. 57:614–7.

(II) Jensen RH, Justesen US, Rewes A, Perlin DS, Arendrup MC. 2014. Echinocandin failure case due to a yet unreported *FKS1* mutation in *Candida krusei*. Antimicrob. Agents Chemother. 58:3550–3552.

(III) Jensen RH, Astvad KMT, Silva LV, Sanglard D, Jørgensen R, Nielsen KF, Mathiasen EG, Doroudian G, Perlin DS, Arendrup MC. 2015. Stepwise emergence of azole, echinocandin and amphotericin B multidrug resistance in vivo in *Candida albicans* orchestrated by multiple genetic alterations. J. Antimicrob. Chemother. 70:2551–2555.

(IV) Jensen RH, Johansen HK, Søes LM, Lemming LE, Rosenvinge FS, Nielsen L, Olesen B, Kristensen L, Leitz C, Dzajic E, Kjaeldgaard P, Astvad KMT, Arendrup MC. 2016. Posttreatment antifungal resistance among colonizing *Candida* isolates in candidemia patients: results from a systematic multicentre study. Antimicrob. Agents Chemother. 60: 1500-08. *Notion: the dataset and presentation of data has been revised for this publication compared to the data presented in this thesis.*

(V) Astvad KMT, Jensen RH, Hassan TM, Mathiasen EG, Thomsen GM, Pedersen UG, Christensen M, Hilberg O, Arendrup MC. 2014. First Detection of TR_{46}/Y121F/T289A and TR_{34}/L98H Alterations in *Aspergillus fumigatus* Isolates from Azole-Naive Patients in Denmark despite Negative Findings in the Environment. Antimicrob. Agents Chemother. 58:5096–101.

(VI) Jensen RH, Hagen F, Astvad KMT, Tyron A, Meis JF, Arendrup MC. 2016. Azole resistant *Aspergillus fumigatus* in Denmark: a laboratory based study on resistance mechanisms and genotypes. Clin. Microbiol. Infect. 22: 570.e1-9.

ABBREVIATION LIST

Abbreviation	Stands for
DNA	Deoxyribonucleic acid
AA	Amino acid
SNP	Single nucleotide polymorphisms
Mutation	Corresponds to changes in the DNA
Alteration/change	Amino acid substitutions in the protein
GENE vs PROTEIN	The gene is *ITALICISED* while PROTEINS are not.
LOH	Loss of heterozygosity, the transition from heterozygous to homozygous of a single mutation or an entire gene/chromosome
GOF	Gain of function, relates to mutations, which renders a gene constitutively expressed
FKS1 and *FKS2*	Genes encoding *FKS1* and *FKS2*, subunits of β-1,3-glucan synthase
ERG11	Gene encoding *ERG11* protein (lanosterol 14-α demethylase in Candida)
ERG2	Gene encoding *ERG2*, C8 isomerase
ERG3	Gene encoding *ERG3*, C5 desaturase
ERG5	Gene encoding *ERG5*, C22 desaturase
ERG6	Gene encoding *ERG6*, Δ[24] sterol C- methyltransferase
CYP51A	Gene encoding *CYP51A* (lanosterol 14-α demethylase in *Aspergillus*)
MRR1	Gene encoding *MRR1* a regulator of MDR genes
MDR1	Gene encoding the Major facilitator *MDR1* (drug efflux pump)

CDR1	Gene encoding *CDR1* ATP-binding cassette (ABC) transporter 1 (drug efflux pump)
CDR2	Gene encoding *CDR2* ATP-binding cassette (ABC) transporter 2 (drug efflux pump)
TAC1	Gene encoding Transcriptional activator of CDR genes
UPC2	Gene encoding *UPC2* a zink cluster transcription factor (regulator of *ERG11*)
MLST	Multilocus sequence typing, genotyping method (most Candida spp.).
STRAf	Short Tandem Repeat *Aspergillus fumigatus*, genotyping method
Pseudo-outbreak	The occurrence of an increased number of positive tests in the laboratory, which does not correlate with clinical findings
ECDC	European Centre for Disease Control
EUCAST	European Committee for Antimicrobial Susceptibility Testing
MIC	Minimal inhibitory concentration
ECOFF	Epidemiological cut-off
AFST	Antifungal Susceptibility Testing
FLU	Fluconazole
VRC	Voriconazole
ITC	Itraconazole
POS	Posaconazole
AMB	Amphotericin B
CAS	Caspofungin
ANI	Anidulafungin
MICA	Micafungin

PART I: INTRODUCTION AND SCOPE

1.1 Drug resistance is associated with treatment failure

The emergence of drug resistant microbes is an inevitable drawback of drug exposure and a true illustration of Charles Darwin's evolution concept "natural selection" [1]. It is drug-induced selection pressure, which eliminates susceptible microbes and allows survival of resistant strains rather than extinction. Thus, resistance is, for the organism, anything but futile and it is self-evident that when involved in microbial infections, drug resistance is highly undesirable and may contribute to treatment failure (Figure 1).

Increased resistance rates, limited therapeutic options and drug resistant microbes, evolved in the environment, displaying cross-resistance to clinical drugs further substantiates this as a serious public health concern [2].

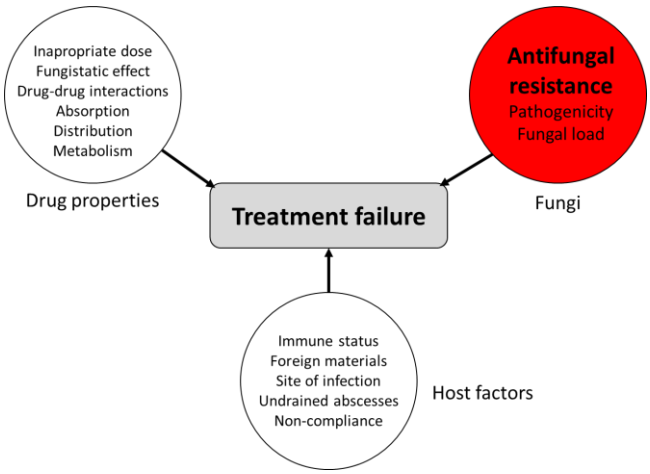


Figure 1. Three factors potentially contributing to treatment failure. When present, antifungal drug resistance may be a significant cause of treatment failure in patients suffering from severe fungal infections.

1.2 Conceptual understanding, antifungal drugs and resistance

The microbes studied in this thesis belong to the *Ascomycetes*, which comprise the most significant fungal pathogens causing critical invasive infections in immunocompromised patients [3, 4]. Treatment of such fungal infections is done by either (or a combination) of the three major antifungal drug classes; azoles, echinocandins and polyenes. Resistance to one drug class clearly challenges treatment due to the limited therapeutic options. Two general terms of resistance will be clearly defined; intrinsic resistance (also known as primary resistance) and acquired resistance (secondary resistance) [5]. Intrinsic resistance is on species-level where certain fungal species display inherited reduced susceptibility to a drug class (Figure 2).

Figure 2. Intrinsic resistance. Left, arbitrary scenario of a polyfungal population consisting of five different species; A, B, C, D and E. A is susceptible to all drug classes, while B is resistant to drug class 1, C is resistant to drug class 2, D is resistant to drug class 3 and E is multidrug resistant. Right, real life panel of species with reduced susceptibility to either of three antifungal drugs. *Candida auris* may display inherently reduced susceptibility to all three antifungals [6].

On the other hand, exposing a susceptible fungus to an antifungal drug can eventually lead to the acquisition of resistance (Figure 3), thus within a susceptible population; one resistant mutant evolves and survives (scenario I). Another route is that within a population, one resistant mutant has spontaneously evolved (scenario II) and antifungal exposure enables this mutant to proliferate rather than the wild-type siblings. However, antifungal resistance often comes with a fitness cost, thus in the absence of an antifungal selection pressure, this mutant will likely vanish from the population (scenario III). Still, in some cases, as we shall see for *Aspergillus fumigatus*, some mutants are equally fit and will persist along with wild-type isolates unaffected by the presence or absence of antifungal selection (scenario IV).

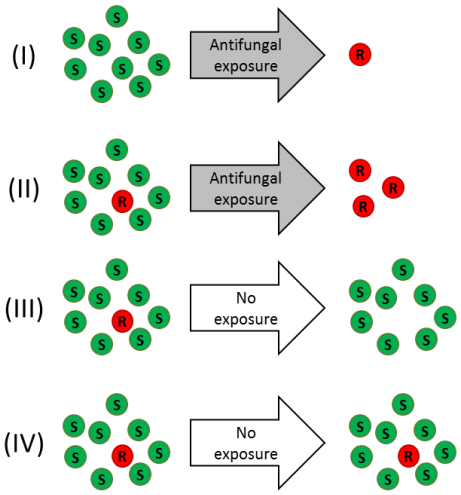


Figure 3. Resistance selection. Acquired drug resistance upon selection and equivalent events when selection is abolished. Stable resistant mutants may rarely occur, which are able to proliferate along with wild-type siblings even in the absence of antifungal exposure.

The underlying molecular mechanisms responsible for resistance depends on the antifungal drug class, to which resistance is observed, and this is tightly correlated to the different modes of actions.

1.2.1 Azoles, mode of action

Clinical azoles is the largest drug class in the management of fungal infections, and they act intracellularly by binding and inhibiting a key enzyme in the ergosterol pathway; lanosterol 14-α-demethylase a cytochrome P450 enzyme (named *ERG11* or *CYP51A* depending on the fungus) (Figure 4) [7]. Ergosterol is the main stabilising component in the fungal cell membranes and thus, an obstruction of the ergosterol synthesis pathway leads to cell membrane stress and growth inhibition [8]. There are several azoles in play, where fluconazole is mainly used for the treatment of *Candida* infections, second generation triazoles such as voriconazole, itraconazole, posaconazole and isavuconazole are primarily used for mould infections [9, 10].

1.2.2 Echinocandins, mode of action

Caspofungin, anidulafungin and micafungin are the current licensed echinocandins used and serve as first-line therapy of invasive *Candida* infections [9]. While primarily fungicidal against yeasts, echinocandins are fungistatic against moulds where they inhibit the growing tips of the hyphae [11]. Echinocandins act by interfering with a subunit of the membrane integrated β-1,3-glucan synthase (*FKS1*) and thereby inhibiting the synthesis of β 1,3 glucans, which are a major component of the fungal cell wall (Figure 4) [12]. This mode of action may explain the effect of echinocandins in the treatment of *Candida* biofilm where the major component in the extracellular matrix is β-1,3-glucans [13].

1.2.3 Polyenes, mode of action

Amphotericin B (AMB) and nystatin are the two licenced polyenes in Denmark, AMB for the treatment of invasive fungal infections and the latter for topical use. AMB acts by binding of ergosterol in the cell membrane leading to membrane instability through sequestering and pore formation, which results in cell death (Figure 4). AMB comes in several lipid formulations but may still be associated with some toxicity primarily due to cross-reaction to human cholesterol (structurally similar to ergosterol) [14]. The drug is acknowledged to show high efficiencies against disseminated fungal infections and is superior in the management of rare fungal infections (e.g. mucormycosis) due do the broad spectrum of action [15, 16].

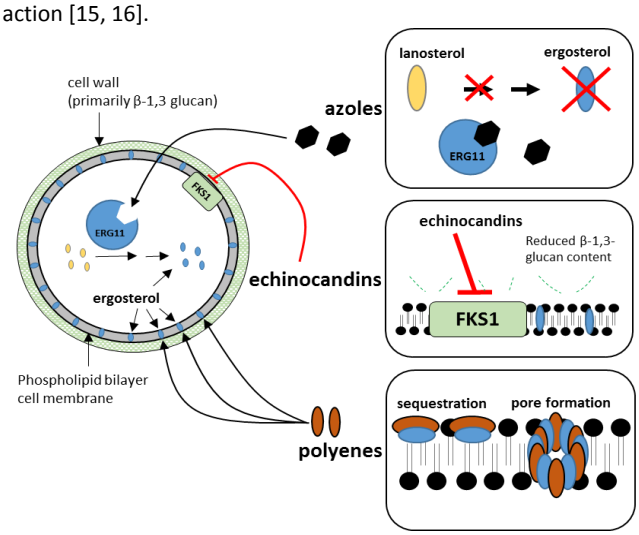


Figure 4. Mechanisms of the three antifungal drug classes. Azoles inhibit *ERG11* and the ergosterol biosynthesis. Echinocandins inhibit *FKS1* and thereby the synthesis of cell-wall β-1,3 glucan and finally polyenes bind ergosterol causing cell membrane instability through sequestration and pore formation.

Structures of the primary antifungal drugs are provided in supplementary reading (S.1 Antifungal drug structures). Each drug displays different pharmacodynamics and pharmacokinetics and although such attributes have been carefully scrutinised, the therapeutic management of a given infection still requires consideration of other parameters, such as the site of infection, severity of infection as well as the infectious agent [9, 10, 17]. Biochemical tests such as serum concentration assays for the mould active azoles enable close monitoring of the drug levels in the blood and ensure that the recommended concentrations are reached [18]. The importance of this is underlined by the fact that sub-optimal concentrations may enable resistance development and lead to clinical failure while too high levels may often be associated with toxicity. Moreover, the site of infection may limit the accessibility of the drug and indeed the abdominal reservoir has been shown to be a niche for resistance development to echinocandins [19]. Undoubtedly, positive cultures of the infectious agent remains invaluable for the optimal therapeutic management because susceptibility testing of the organism becomes available.

1.2.4 Susceptibility testing, breakpoints and interpretation of resistance

Several in vitro assays have been developed in order to test the susceptibility of an organism provided as the minimal inhibitory concentration (MIC); the concentration of a drug, which is required to kill or inhibit growth of the fungus. The subgroup Antifungal Susceptibility Testing (AFST) of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) has established international reference protocols in order to normalise the methodology by which susceptibility is measured. Furthermore, EUCAST has set forth a species-specific approach for the most prevalent pathogenic yeasts (E.Def 7.2) [20] and moulds (E.Def 9.2) [21] and established clinical breakpoints. Such breakpoints can be used to determine whether an organism is susceptible (S), intermediate (I) or resistant to a drug, which in turn may be translated to clinical susceptibility. Susceptibility testing of wild-type populations results in a normal distribution of the MIC data as illustrated in Figure 5 and illustrate the abovementioned definitions of susceptibility.

Two steps above the modal MIC is often (but not always) defined as the epidemiological cut-off (ECOFF) value and although it may not translate to the clinical breakpoints, ECOFFs may be carefully applied in the absence of established breakpoints. Isolates classified as resistant should be treated with an alternative drug class [9, 10]. In this thesis, susceptibility was performed by the EUCAST methodology and interpreted with EUCAST breakpoints or ECOFFs as provided in Table 1 (adapted from http://www.eucast.org/clinical_breakpoints). The CLSI breakpoints were applied for E-test susceptibility testing and indicated when used [22, 23].

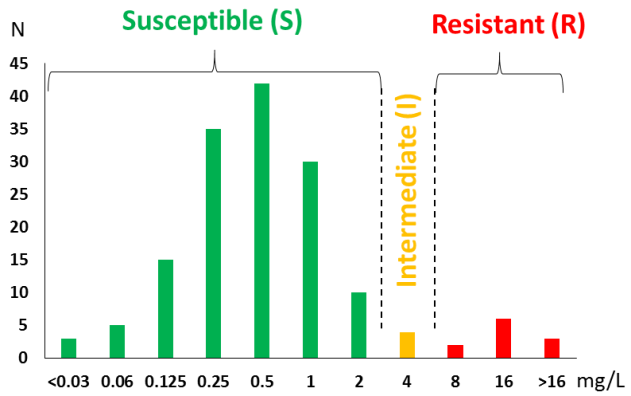


Figure 5. Fluconazole MICs for *Candida albicans*. The X-axis defines the MIC values and illustrates the three classifications susceptible (S), intermediate (I) and resistant (R). This indicates a normal distribution, and shows the intermediate step and MIC concentrations where the organism is regarded resistant. The breakpoint provided here corresponds to the suggested fluconazole breakpoint by EUCAST for *C. albicans*.

Table 1. Applied EUCAST breakpoints for the discrimination of susceptible and resistant isolates.

	MIC breakpoints (mg/L)															
	FLU		VRC		ITC		POS		ANI		MICA		CAS		AMB	
Species	**S ≤**	**R >**	**S ≤**	**R >**	**S ≤**	**R >**	**S ≤**	**R >**	**S ≤**	**R >**	**S ≤**	**R >**	**S ≤**	**R >**	**S ≤**	**R >**
C. albicans	2	4	0.12	0.12	0.06	0.06	0.06	0.06	0.03	0.03	0.016	0.016	NE	NE	1	1
C. glabrata	0.002	32	NE	NE	NE	NE	NE	NE	0.06	0.06	0.03	0.03	NE	NE	1	1
C. krusei	R	R	NE	NE	NE	NE	NE	NE	0.06	0.06	0.25[2]	NE	0.25[1]	0.5[1]	1	1
C. tropicalis	2	4	0.12	0.12	0.12	0.12	0.06	0.06	0.06	0.06	0.03[2]	NE	0.25[1]	NE	1	1
A. fumigatus	R	R	1	2	1	2	0.12	0.25	NE	NE	NE	NE	NE	NE	1	2

FLU, fluconazole, VRC, voriconazole; ITC, itraconazole; POS, posaconazole; ANI, anidulafungin; MICA, micafungin; CAS, caspofungin; AMB, amphotericin B. R, resistant; NE: not established.

[1]Revised CLSI breakpoints used for C. tropicalis in Paper I and C. krusei in Paper II.

[2]The applied breakpoints were not established by EUCAST. Arguments for the chosen breakpoints were described in Paper I and Paper II for C. tropicalis and C. krusei, respectively.

1.3 Ploidy and pathogenicity

The organisms studied here are a heterogeneous population both in terms of ploidy (number of sets of chromosomes in the cells), pathogenicity and intrinsic susceptibility, which is illustrated in Figure 6 [18, 24–26]. Assessment of heterozygosity in diploid organisms was carried out based on scrutinised interpretation of Sanger sequencing results and have been further illustrated in supplementary reading (S.2 Sequence interpretation and ploidy).

1.4 Scope and structure of the thesis

In Denmark, acquired antifungal resistance has been a rare phenomenon both in the Danish fungaemia programme (initiated in 2003) and among *Aspergillus* infections [18, 27]. Still, the lack of susceptibility testing and/or referral of isolates may have contributed to an underestimation of this extent. Despite receiving increased worldwide attention, little was known in Denmark and thus, elucidating the Danish antifungal resistance epidemiology seemed warranted. Accordingly, we set forth to investigate the prevalence and underlying molecular mechanisms of antifungal resistance among the most clinically relevant fungal yeast (*Candida* species) and mould (*Aspergillus fumigatus*) in Denmark. Moreover, for *A. fumigatus* to draw a link to environmentally derived resistance and finally to discuss the aspects of these apparent concerns. This thesis is divided into four sections focusing on:

- Part I: Background
  - Provide a conceptual understanding of antifungal drugs and resistance.
- Part II: Resistance in *Candida*
  - Describe the underlying molecular resistance mechanisms in *Candida* supported by case studies.
  - Investigate the prevalence of resistance in colonising *Candida* among *Candida*emia patients post treatment.

- Part III: Resistance in *A. fumigatus*
  - Outline the current situation on azole resistance in *Aspergillus fumigatus* in Denmark.
  - Describe azole resistance in *A. fumigatus* potentially derived from environmental fungicide use.
- Part IV: Discussion
  - Evaluate the impact of these findings and reflect on future research needs related to the field of invasive fungal infections.

Paper I illustrated the two-step genetic event leading to echinocandin resistance in *Candida tropicalis*. Paper II described an intrinsically fluconazole resistant *Candida krusei* with acquired echinocandin resistance.

Paper III presented a clinical case with a gradual development of multidrug resistance in a series of clinical *C. albicans* isolates and sought to describe the complex genetic resistance mechanisms Paper IV investigated the prevalence of antifungal resistance among colonising *Candida* isolates in *Candida*emia patients exposed to antifungal drugs.
Paper V presented fatal cases involving azole resistant *A. fumigatus* possibly derived from the environment. Paper VI sought to clarify the current resistance epidemiology of clinical and environmental *A. fumigatus* isolates in Denmark and studied accumulated genotyping data in relation to the potential of clonal expansion.

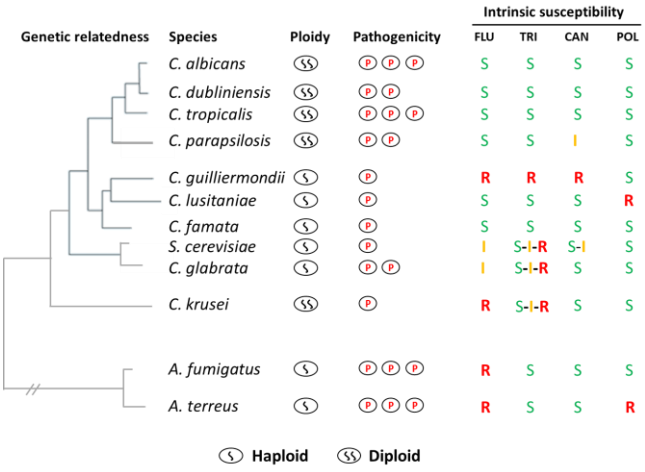


Figure 6. Characteristics of important fungal pathogens. Ploidy of the organisms as well as the level of pathogenicity and intrinsic susceptibility patterns of each wild-type population is shown. FLU, fluconazole, TRI, triazoles, CAN, echinocandins and POL, polyenes.

PART II: RESISTANCE IN THE OPPORTUNISTIC YEAST PATHOGEN *CANDIDA*

2.1 Candida epidemiology

Most fungal bloodstream infections are caused by Saccharomycetes yeasts *Candida* species, which are commensals of the human body primarily residing at mucosal surfaces such as the oral cavity, gastrointestinal tract and the vagina [18]. Risk factors for acquiring a *Candida* bloodstream infection (Candidaemia) include surgery (especially those of the gastrointestinal tract), immunosuppression, haematological malignancies and introduction of foreign material (catheters and prostheses, which enable biofilm formation) [28]. The mortality rate of Candidaemia is in the range of 30-40 % although the attributable mortality rates may be lower [29]. Several studies have documented that catheter removal and early initiation of antifungal treatment significantly increased survival rates in patients suspected with Candidaemia [30]. The Danish fungaemia surveillance network managed by the Mycology Unit at Statens Serum Institut monitors the epidemiology of fungal bloodstream infections in Denmark. Around 500 cases have been found annually corresponding to an incidence of 10/100,000) with about 98% caused by *Candida* species [31]. Obviously, knowledge on national epidemiology is essential in order to issue relevant therapeutic guidelines and in Denmark, the epidemiology has gradually shifted over the last decade. Thus, the intrinsically susceptible *Candida albicans* accounts for the majority 50% of all cases, while *Candida glabrata*, intrinsically less susceptible to fluconazole, has risen to approximately one third of all cases (Figure 7) [18].

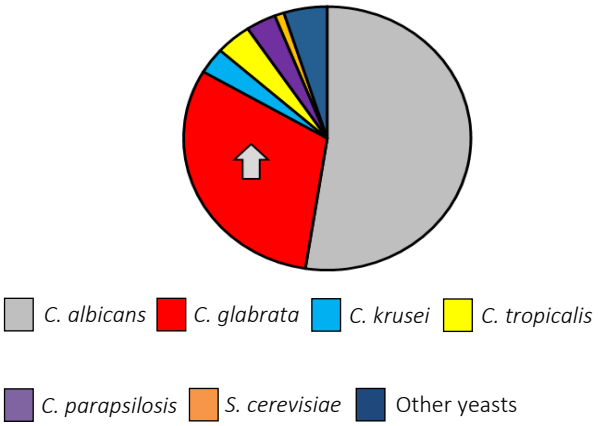


Figure 7. *Candida*emia species distribution over a 12-month period. A total of 471 *Candida* isolates were collected from *Candida*emia patients over a 12-months period in 2013-2014. This distribution is only a close approximation of the current *Candida*emia species distribution as some isolates from 2014 were referred with delay and not included here. Arrow indicates an increasing prevalence of *C. glabrata* over recent years.

The diagnosis of invasive candidiasis relies primarily on a positive blood culture, although clinical manifestations combined with several biomarker assays and microscopy, collectively support such diagnosis [18]. Still, molecular methods are increasingly acknowledged as a rapid and efficient alternative, because it targets DNA and pose superior sensitivity compared to the low-sensitive culturing [32, 33]. Certainly, the timing of diagnosis is correlated to outcome and rapid tests may improve the prognosis of such patients. Moreover, species identification plays an important role due to the large variations in susceptibility and the increasing prevalence of species less susceptible to fluconazole. The changing epidemiology as well as superior efficiencies, recent guidelines in Denmark and other countries with similar epidemiology have altered first-line therapy to echinocandins [31, 34]. This however, is associated with other concerns because resistance to this drug class is notorious to rapidly emerge during echinocandin treatment [35].

2.2 Echinocandin resistance and FKS variations in Candida (Paper I-II)

Both intrinsic and acquired resistance to echinocandins have almost solely been linked to variations of two specific hot-spot

regions of the *FKS1* protein [13, 36]. In *C. glabrata* and Saccharomyces cerevisiae, *FKS2* is a homologous gene in which, especially for *C. glabrata*, mutations have also been shown to confer echinocandin resistance [37]. Despite that the crystal structure remains to be solved, in silico hydrophobicity analysis of the *FKS1* protein sequence has been performed and the proposed transmembrane protein is illustrated in Figure 8 [37]. The location of the two hot spots indicated in the figure may not be exact but it does suggest the external location of the two *FKS1* hot spots and thus that the echinocandins do not enter the cell [38]. This may explain the absence of drug-efflux related resistance mechanisms for the echinocandins.

Thus, intrinsic or acquired variations of the hot spot regions lead to structural changes of *FKS1*, some of which reduce echinocandin affinity. Selection for echinocandin resistance in vivo has been demonstrated for several *Candida* species including *C. albicans* [39–43], *C. glabrata* [43–47] and *C. krusei* [43, 48, 49] and Table 2 provides an overview of amino acids in *FKS1* and *FKS2* associated with resistance in different *Candida* species.

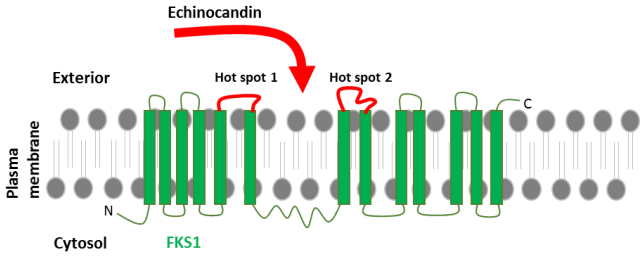


Figure 8. Proposed structure of the *FKS1* transmembrane protein. The transmembrane helices are shown as green barrels and the suggested hot spot regions coloured red. One study has suggested a third hot spot near and downstream of hot spot 1 but the actual role of this region in relation to echinocandin susceptibility is not fully elucidated [37].

Table 2. *FKS* hot spot overview of *Candida* species. Amino acid (AA) sequence of *FKS1* and *FKS2* hot spots in relevant *Candida* species in relation to echinocandin resistance updated from [50]. Species with documented acquired AA variants associated with echinocandin resistance are listed first followed by species with intrinsic AA variants potentially involved in reduced echinocandin susceptibility.

		FKS1 and FKS2 amino acid sequences		
Species (FKS)	**EUCAST BP[E] mg/L**	**Hot spot 1 (FLTLSLRDP)**	**Hot spot 2 (DWIRRYTL)**	**References**
C. albicans (FKS1)	0.03	641-FLTLSLRDP	1357-DWIRRYTL	[51–54]
C. glabrata (FKS1)	0.06	625-FLILSLRDP	1340-DWVRRYTL	[52, 55, 56]
C. glabrata (FKS2)	0.06	659-FLILSLRDP	1374-DWIRRYTL	[52, 55–57]
C. krusei (FKS1)**	0.06[#]	655-FLILSIRDP	1364-DWIRRYTL	[58–62]
C. tropicalis (FKS1)*	0.06	76-FLTLSLRDP	792-DWIRRYTL	[55, 60, 63, 64]
C. dubliniensis (FKS1)	0.03	641-FLTLSLRDP	1357-DWIRRYTL	[65], this study
C. lusitaniae (FKS1)*	(0.06)	634-FLTLSLRDP	NA*-DWIRRYTL	[66], this study
C. kefyr (FKS1)*	(0.03)	54-FLTLSLRDP	769-DWVRRYTL	[67, 68], this study
C. parapsilosis (FKS1)	4	652-FLTLSLRDA	1369-DWIRRYTL	[69]
C. metapsilosis (FKS1)*	(4)	104-FLTLSLRDA	821-DWIRRYTL	[69]
C. orthopsilosis (FKS1)*	(4)	39-FLTLSLRDA	756-DWVRRYTL	[69]
C. guilliermondii (FKS1)	(4)	632-FMALSLRDP	1347-DWIRRYTL	[51]
S. cerevisiae (FKS1)	(1)	639-FLVLSLRDP	1353-DWVRRYTL	[37, 70, 71]
S. cerevisiae (FKS2)	(1)	658-FLILSLRDP	1372-DWVRRYTL	[37, 70, 71]
C. lipolytica (FKS1)	NA	662-FLILSLRDP	1387-DWIRRCVL	[69]

Intrinsic or acquired amino acid (AA) variants in association with echinocandin susceptibility are in bold colour font:

X *"strong R", associated with high resistance when altered. Involves stop codons and amino acid deletions.*

X *"weak R", medium or little impact on susceptibility when altered.*

X *natural AA variant associated with intrinsic resistance.*

X *natural AA variant with no suspected effect on susceptibility.*

X *natural AA variant with unknown effect on susceptibility.*

EBreakpoints or ECOFFs are indicated. ECOFFS based on MICs of Danish blood isolates, peak MIC + 2 dilution steps.

Underlined amino acids have been discovered as variants associated with resistance in Danish clinical isolates.

**Accurate annotation remains unavailable.*

***F645L and L701M outside hot-spot 1 are suggested to affect echinocandin susceptibility in C. krusei.*

#micafungin (but not anidulafungin) ECOFF elevated for C. krusei (0.25 mg/L) compared to C. albicans (0.015 mg/L) and C. glabrata (0.03 mg/L).

As indicated in the table above, C. parapsilosis, C. metapsilosis, C. orthopsilosis and C. guilliermondii all harbour intrinsic *FKS1* variants, which have been shown to be responsible for intrinsic reduced echinocandin susceptibility [69]. Multiple *FKS1* changes have been detected and especially the amino acid corresponding to S645 in *C. albicans* is prone to alterations leading to significant echinocandin resistant phenotypes. Yet, resistance to echinocandins may come with a fitness cost because the altered *FKS1* protein in turn can display reduced catalytic activity and thus reduced biosynthesis of the cell wall components [72].

Among the total of 45 Danish echinocandin resistant clinical isolates accumulated since 2008 (Table 3), *C. glabrata* accounted for 56% (25/45) of all echinocandin resistance cases, while *C. albicans* only comprised 16% (7/45). These numbers contrast the prevalence in the *Candida*emia settings where *C. glabrata* only constitutes one third.

Table 3. *FKS* profiles of Danish clinical *Candida* with reduced echinocandin susceptibilities.

				EUCAST(and Etest MICs (mg/L)					*FKS* hot spot mutations
Patient ID	**Species**	**Specimen**	**Date**	**ANI**	**MICA**	**VOR**	**FLU**	**AMB**	**AA substitution (no. in *C. albicans*)**
SSI-OV-30	*C. albicans*	Oral swab	14.11.13	0.03	0.03	≤0.03	≤0.125	0.5	F641L
SSI-OV-56	*C. albicans*	Blood	18.2.15	0.25	1	≤0.03	≤0.125	0.125	S645P
SSI-OV-8	*C. albicans*	Urine cath.	3.6.14	0.5	>1	≤0.03	0.5	0.5	S645P
SSI-OV-20	*C. albicans*	Colon	06.5.11	0.5	na	0.125	≥16	>32	S645P^{H}/V661F^{H}***
SSI-OV-18	*C. albicans*	Oral swab	29.4.14	0.06	0.25	≤0.03	2	0.25	R647G
SSI-OV-38	*C. albicans*	Oral swab	29.8.13	0.125	0.06	≤0.03	≤0.125	0.38	D648V
SSI-OV-57	*C. albicans*	Blood	3.9.14	0.06	0.06	≤0.03	≤0.125	0.38	R1361H
SSI-OV-50	*C. dubliniensis*	Blood	27.7.8	0.125	na	≤0.03	≤0.125	0.02	F641S (F641)****
SSI-OV-41	*C. dubliniensis*	Blood	20.11.8	0.5	na	≤0.03	≤0.125	0.25	S645P (S645)
SSI-OV-26	*C. glabrata*	Oral swab	31.5.13	0.125	0.015	≤ 0.03	1	1	F659L (F641)
SSI-OV-20	*C. glabrata*	Oesoph.	25.2.11	0.125	na	4	>16	0.5	F659L (F641)
SSI-OV-27	*C. glabrata*	Blood	24.8.12	0.125	0.03	4	>16	0.03	F659S (F641)
SSI-OV-51	*C. glabrata*	Blood	5.2.9	0.50	na	0.25	8	2	F659S (F641)
SSI-OV-37	*C. glabrata*	Blood	9.9.14	0.25	0.06	≤0.03	0.25	0.25	F659C (F641)
SSI-OV-1	*C. glabrata*	Blood	6.8.11	0.125	0.06	2	>16	0.5	F659-DEL (F641)
SSI-OV-49	*C. glabrata*	Blood	30.5.13	0.125	0.125	2	>16	0.5	F659-DEL (F641)
SSI-OV-46	*C. glabrata*	Blood	3.12.12	0.25	0.25	0.125	4	0.5	F659-DEL (F641)
SSI-OV-11	*C. glabrata*	Oral swab	29.8.13	0.5	1	0.25	8	0.5	F659-DEL (F641)
SSI-OV-9	*C. glabrata*	Blood	30.4.13	>1	>1	0.5	16	0.25	F659-DEL/L712-STOP (F641/L728)
SSI-OV-35	*C. glabrata*	Trach	21.12.11	2	na	4	>16	0.5	S629P (S645)
SSI-OV-39	*C. glabrata*	Blood	29.6.12	0.03	0.125	≤0.03	2	0.5	S663F (S645)
SSI-OV-55	*C. glabrata*	CVC-tip	31.10.14	0.125	0.06	0.125	8	0.5	S663P (S645)
SSI-OV-22	*C. glabrata*	Oral swab	23.1.14	0.5	0.125	2	>16	0.5	S663P (S645)
SSI-OV-10	*C. glabrata*	Oral swab	24.10.13	0.5	0.25	0.06	4	0.5	S663P (S645)
SSI-OV-40	*C. glabrata*	Blood	23.7.13	0.25	0.5	2	>16	0.125	S663P (S645)
SSI-OV-42	*C. glabrata*	Trach.	14.2.14	0.25	0.5	0.125	4	0.5	S663P (S645)
SSI-OV-1	*C. glabrata*	Blood	6.8.11	0.5	0.5	4	>16	0.5	S663P (S645)
SSI-OV-15	*C. glabrata*	Oral swab	9.12.13	1	1	1	>16	1	S663P (S645)
SSI-OV-29	*C. glabrata*	Oral swab	22.4.15	1	1	0.125	8	0.125	S663P (S645)
SSI-OV-16	*C. glabrata*	Blood	23.9.14	>1	>1	2	>16	1	S663P (S645)
SSI-OV-39	*C. glabrata*	Blood	12.4.13	1	1	≤0.03	2	1	S663F/L630Q (S645/L646)
SSI-OV-24	*C. glabrata*	Blood	23.8.14	0.125	0.06	1	>16	0.5	D666E (D648)
SSI-OV-2	*C. glabrata*	Blood	17.10.12	0.25	0.03	0.125	8	0.5	P667T (P649)
SSI-OV-53	*C. glabrata*	Blood	28.2.15	0.03	0.06	4	>16	0.25	K1357M (V1340)
SSI-OV-13	*C. krusei*	Blood	16.8.13	>1	>1	0.25	>16	0.5	D662Y (D648)**
SSI-OV-21	*C. krusei*	BAL	17.12.14	0.06	0.125	0.125	>16	0.5	L701M (L687)
SSI-OV-5	*C. krusei*	Blood	30.10.14	0.125	0.125	0.5	>16	0.5	L701M (L687)
SSI-OV-48	*C. krusei*	Blood	21.11.13	0.125	0.125	0.5	>16	1.5	L701M (L687)
SSI-OV-4	*C. krusei*	Blood	2.1.15	0.125	0.25	0.25	>16	0.125	L701M (L687)
SSI-OV-52	*C. krusei*	Oral swab	25.6.13	0.125	0.25	1	>16	1	L701M (L687)
SSI-OV-23	*C. tropicalis*	Blood	4.11.12	0.125	0.06	0.06	1	0.5	F76L (F641)
SSI-OV-44	*C. tropicalis*	Blood	12.2.12	0.5	2	≤0.03	≤0.125	1	S80P (S645)
SSI-OV-25	*C. tropicalis*	Oral swab	1.4.11	0.5	>1	0.125	2	1	S80P (S645)*
SSI-OV-17	*C. lusitaniae*	Urine cath.	20.5.15	>1	>1	≤0.03	0.25	0.03	S638F (S645)
SSI-OV-45	*C. kefyr*	Blood	15.11.14	>1	>1	≤0.03	0.5	1	S645L/I1347L/V1330I**** (S645)

HHeterozygous variant.
**Presented in Paper I*
***Presented in Paper II*
****Presented in Paper III*
*****to our knowledge, first description in this species and potentially conferring echinocandin resistance*

The high occurrence of *FKS* mutants in *C. glabrata* indicated a strong capacity of this species to acquire echinocandin resistance. Furthermore, the amino acid corresponding to S645 in *C. albicans* was the target of 44% (20/45) of all detected *FKS* variants associated with resistance and F641 accounted for 29% (13/45) (Figure 9).

As indicated in Table 3, a few specific cases were presented individually in Paper I-III. In Paper I [64], three C. *tropicalis* isolates were obtained from a patient with acute lymphoblastic leukaemia obtained within 9 weeks of caspofungin treatment. The first isolate was susceptible, while the second and third isolates were echinocandin resistant (Table 4). Multilocus sequence typing

(MLST) indicated genetic relatedness [73] and *FKS1* sequencing showed a gradual development of a homozygous mutation, which led to the AA substitution S80P corresponding to S645 in *C. albicans*.

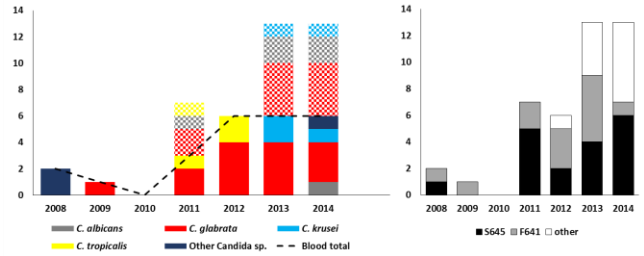


Figure 9. Historical representation of *FKS* variants in Danish *Candida* isolates since 2008. Y-axis, number of isolates. Left panel illustrates the number of isolates with acquired *FKS* mutations. Colours represents species, chequered patterns are isolates not obtained from blood and the broken line indicates the number of *FKS* mutants among blood isolates. Right panel illustrates the number of isolates with AA loci, which have been altered represented by the two most frequent sites corresponding to S645 and F641 in *C. albicans* and other.

Table 4. *Candida tropicalis* isolate overview, Paper I. Origins, resistance mechanisms, genotypes and susceptibility data (reproduced with permission from the publisher ASM).

				MIC (mg/L)[a]						
				EUCAST (EDef 7.1)					Etest	
Isolate*	Collection date (day.mo.yr)	*FKS1* Resistance mechanism	Allelic profiles according to the pubMLST database (*ICL1-MDR1-SAPT2-SAPT4-XYR-ZWF*)	POS	ANI	VRC	ITC	FLU	AMB	CAS
#1[BC]	19.12.10	WT	16-20-4-10-25-5	≤0.03	≤0.03	≤0.03	0.06	1	0.50	0.125
#2[BC-H]	5.03.11	S80S/P	16-20-4-10-25-5	≤0.03	0.25	≤0.03	≤0.03	0.25	0.50	>32
#3[CO]	18.03.11	S80P	16-20-4-10-25-5	0.25	0.5	0.125	0.125	2	1	>32
REF-1[b]	8.07.10	WT	1-7-4-6-2-4	≤0.03	≤0.03	≤0.03	≤0.03	≤0.125	1	N/A
REF-2[b]	23.01.11	WT	1-3-1-7-2 (99.7 %)-1	≤0.03	≤0.03	≤0.03	0.125	0.5	0.5	0.125

**origin of sample. BC, blood culture; BC-H, Blood culture obtained via an intravenous Hickman catheter; CO, Cavum oris. WT, wild-type.*
aANI, anidulafungin; MICA, micafungin; POS, posaconazole; VRC, voriconazole; ITC, itraconazole; FLU, fluconazole; AMB, amphotericin B; CAS, caspofungin.
bSusceptible reference isolate from unrelated patient included for comparison.

The S80S/P variant (heterozygous) was described previously in association with echinocandin resistance [63, 74] but the stepwise in vivo development of the S80P variant (homozygous) had to our knowledge not been shown before. The homozygous mutation could be associated with fitness costs as observed for *C. albicans* [72]. Yet, a potentially higher level of resistance for a homozygous variant, also seen in *C. albicans* [52, 75] could ultimately explain, why this loss-of-heterozygosity (LOH) did occur [76].

In Paper II [59], a breakthrough infection of a highly echinocandin resistant *C. krusei* isolate was presented. The resistant isolate harboured a novel *FKS1* variant D662Y, corresponding to D648Y in *C. albicans* [60], from a patient previously exposed to fluconazole (2 months) and caspofungin (14 days). The patient died on day 25 from cerebral infarction and fungal infection. The most prominent finding was the relatively strong impact, which this amino acid substitution may have had on echinocandin susceptibility in comparison with the equivalent variant found in *C. albicans* (Table 5).

Table 5. Strain representation from the *C. krusei* study. Species, *FKS1* profiles and echinocandin susceptibility data for the *C. krusei* isolate and relevant reference isolates (reproduced with permission from the publisher ASM)

		MICs (mg/L) and susceptibility*			
		EUCAST		**Etest**	***FKS1* hot spot 1**
Isolate #	**Species**	**Anidulafungin**	**Micafungin**	**Caspofungin**	**AA no.-sequence**
ATCC6258	*C. krusei*	0.03 S	0.125 WT	0.5 I	655-FLILSIRDP
CPH-T5842	*C. krusei*	>1 R (≥5)	>1 non-WT (≥3)	16 R (6)	655-FLILSI**RY**P
CPH-T53911	*C. albicans*	0.008 S	0.008 S	0.125 S	641-FLTLSLRDP
DPL-1012	*C. albicans*	0.06 R (1)	0.06 R (2)	1 R (2)	641-FLTLSLR**Y**P

**Dilution steps above clinical breakpoints (anidulafungin and caspofungin) or ECOFF (micafungin) are provided in parenthesis. S, susceptible; I, intermediate; R, resistant; WT, wild-type MIC.*
The intrinsic amino acid (AA) isoleucine (I660) uniquely found in the C. krusei FKS1 hot spot region 1 is underlined and the FKS1 substitution is bold.

Similarly to *C. albicans*, the corresponding D632Y substitution in *FKS1* of *C. glabrata* was shown to confer discrete echinocandin MIC elevations [77, 78]. Yet, since an isogenic wild-type susceptible *C. krusei* isolate was not available, it remains to be confirmed whether this single D662Y substitution was solely responsible for the observed high level echinocandin resistance in *C. krusei*.

Indeed, the wild-type population of *C. krusei* isolates does display higher echinocandin MICs, most pronounced for micafungin, compared to *C. albicans*, which could potentially be related with the intrinsic amino acid variation I660, unique for *C. krusei* (confer Table 2). It is acknowledged however, that echinocandin resistance depends both on *FKS1* genotype as well as species, which emphasises the therapeutic challenges when encountering acquired resistance.
While echinocandin resistance remains primarily coupled to changes of *FKS1* and *FKS2*, azole and polyene resistance mechanisms in *Candida* are more complex [79, 80].

2.3 Azole resistance in Candida is often multifaceted

There are four cellular mechanisms, which have been described to be responsible for azole resistance either solely or in interplay and potentially triggered by the stress response protein Hsp90 (Figure 10) [81, 82].

(I) Genetic mutations in the gene encoding *ERG11*, which results in amino acid changes and thus an altered protein structure reducing azole drug affinity [83].
(II) Overexpression of *ERG11*, which results in more *ERG11* proteins and thus higher concentration of the drug is required for inhibition [84].
(III) Increased azole export by upregulated drug efflux transporters *MDR1*, *CDR1* and *CDR2* [85].
(IV) Bypass of the *ERG11* dependent sterol pathway enabled by *ERG3* inactivation (loss-of-function) is a fourth but less common mechanism [86–88].

For resistance mechanism (I), numerous reports have associated amino acid changes in *ERG11* with azole resistance (e.g. A61, Y132, T229, G307, S405, G450 and I471), and the list is continuously expanding [83, 90–93]. Significant amino acid sites have been shown independently or in combination to be associated with reduced fluconazole and/or pan-azole susceptibilities when altered [83]. Still, not all AA substitutions have been validated genetically but a recent study solved the crystal structure of *ERG11* in S. cerevisiae [94], which serves as a model for in silico studies of *C. albicans ERG11* amino acid variations by homology modelling [95].

The genetic regulation of azole resistance is now well characterized. *ERG11* upregulation (mechanism (II)) is linked to specific gain-of-function (GOF) mutations in the zinc-cluster transcription factor encoding gene *UPC2* [96–100], as well as to increases in copy number due isochromosome formation of chromosome 5 [101, 102]. In mechanism (III) GOF mutations in transcription factors *TAC1* (transcriptional activator of CDR genes) and *MRR1* (regulator of MDR genes) upregulate the major drug efflux pumps ABC transporters *CDR1*/*CDR2* and the major facilitator efflux pump *MDR1*, respectively [90, 103–107]. In addition, chromosome 5, on which both *ERG11* and *TAC1* are situated, has been shown to be capable of undergoing transformations, which involves a haploid state of chromosome 5 (loss of a chromosome) and subsequent duplication to restore a diploid and homozygous state (LOH). This may have significant implications because passive heterozygous mutations become homozygous resulting in a higher potential both with regards to structural changes of *ERG11* but also the regulatory effect of *TAC1* [101, 102].
When *ERG11* is inhibited, another protein, *ERG6*, mediates an alternative pathway transforming lanosterol to 14α-methylated sterols (Figure 11). This however involves *ERG3*, which is responsible for the formation of cell toxic 14α-methyl-ergosta-8,24(28)-dien-3β-6α-diol. Thus, when *ERG3* is rendered inactive (resistance mechanism (IV)), suitable sterols are formed as alternatives to ergosterol during azole inhibition of *ERG11* [108, 109].

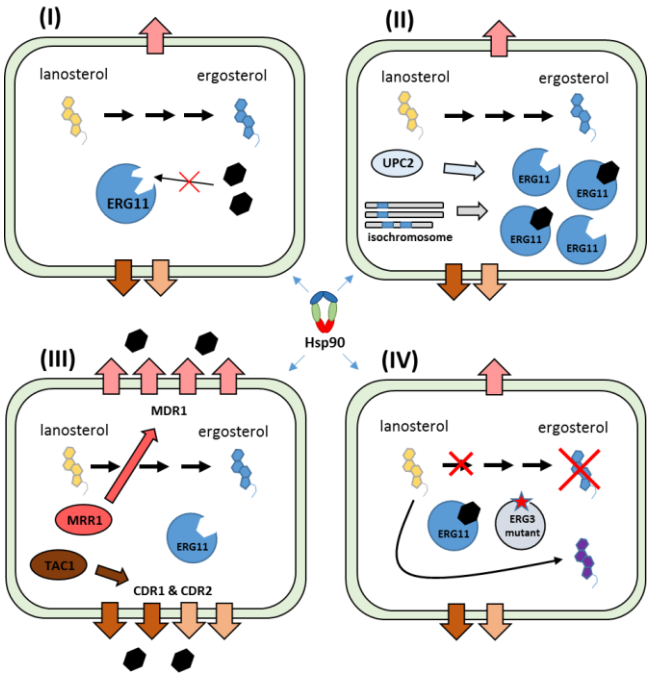


Figure 10. Azole resistance mechanisms in *Candida*. (I) alterations in the *ERG11* protein leading to reduced azole affinity. (II) upregulation of the gene encoding *ERG11*. (III) upregulation of drug-efflux pumps, which reduces the concentration of cytosolic azoles. (IV) *ERG11* independent sterol synthesis pathway is enabled due to *ERG3* inactivation. All four mechanisms may in part have been regulated by a stress response pathway triggered by Hsp90 [89].

While azoles inhibit an early step in the biosynthesis of ergosterol, polyenes bind ergosterol and thus the shared target may explain the occurrence of cross-resistance to polyenes and azoles [110, 111].

2.4 Polyene resistance is associated with ergosterol depletion

Polyenes bind to the primary cell membrane component ergosterol. Thus, in order for the fungus to evade polyenes, the cells are required to alternate the sterol content of the cell membrane to non-ergosterol sterols. Maintaining cell membrane stability in the absence of ergosterol is rarely beneficial for the fungus and is thus often associated with reduced fitness [85, 112]. Despite rare, polyene resistance has been detected and linked to the late obstruction of the ergosterol biosynthesis pathway (Figure 11) [14, 113] involving mutations in *ERG2* [110, 112], *ERG3*, [114–116], *ERG6* [117] or *ERG11* and *ERG5* [118].

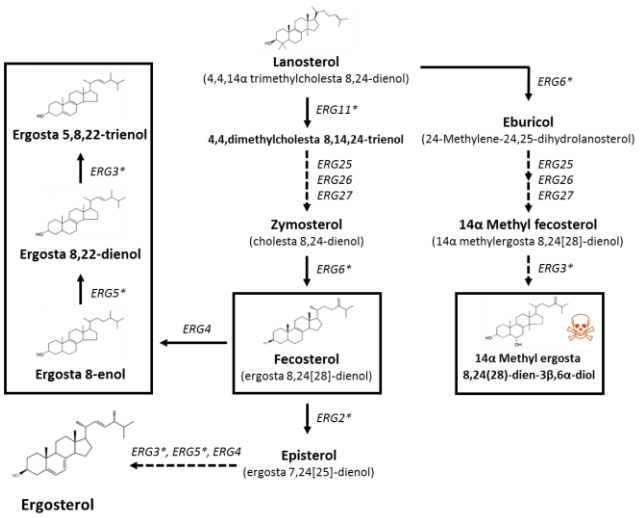


Figure 11. Ergosterol biosynthetic pathway in *C. albicans* from lanosterol to ergosterol. Alternative pathways enable the synthesis of other sterols serving as escape mechanisms when critical stages are inhibited. Asterisk (*) indicates those genes, which have been shown previously to be involved in polyene resistance [110, 112, 114–118].

2.5 Multidrug resistance in C. albicans orchestrated by multiple genetic events (Paper III)

Paper III [119] presented a unique case of serial clinical *C. albicans* from a single patient, developing resistance to azoles, echinocandins and polyenes in a stepwise manner and during several years of antifungal treatment (overview can be found in supplementary reading for Paper III). Assessment of resistance in these isolates involved genotyping (to confirm genetic relatedness) by MLST analysis [120, 121] and sequencing of genes, which have previously been linked to drug resistance. This included *FKS1* for echinocandin resistance [36, 51], *ERG11* [8], *TAC1* and *UPC2* [122] for azole resistance and multiple genes encoding proteins within the ergosterol pathway for polyene resistance. Moreover, characteristics such as gene expression analyses [122, 123] and ergosterol quantitation [7] substantiated the resistance profiles as well as the association between detected genetic changes and phenotypic resistance. All significant findings are presented in Table 6 and are assessed below.

Table 6. Characteristics of nine related and increasingly resistant *C. albicans* isolates. Site and date, susceptibility, gene products and relative gene expression levels for the isolates, P-1 through P-9 (reproduced with permission from the publisher Oxford University Press).

	P-1 (WT)	P-2 (WT)	P-3 (F)	P-4 (F)	P-5 (A)	P-6 (A+E)	P-7 (A+E)	P-8 (MDR)	P-9 (MDR)
Site	Oesophagus^{P}	Oesophagus^{P}	Oropharynx^{C}	Oropharynx^{C}	Oesophagus^{P}	Oesophagus^{P}	Faeces^{C}	Faeces^{C}	Colon biopsy^{P}
Date	25.04.06	11.07.06	28.01.08	01.04.08	21.04.10	17.08.10	10.04.11	10.04.11	06.05.11
FLU$^{7.2}$	0.125	0.25	16	8	>16	>16	>16	>16	16
ITZ$^{7.2}$	≤0.03	≤0.03	≤0.03/4*	≤0.03	16	>4	16	16	>16
VRZ$^{7.2}$	≤0.03	≤0.03	≤0.03/4*	≤0.03	1	0.5	0.25	0.125	0.125
POS$^{7.2}$	NA	NA	≤0.03/4*	≤0.03	>4	>4	4	4	0.5/4*
ANI$^{7.2}$	NA	NA	NA	0.015	0.015	0.25	1	1	0.5
CAS^{ET}	0.06	0.25	0.25	0.25	0.50	>32	>32	>32	>32
AMB^{ET}	0.25	0.5	0.38	0.5	0.5	0.5	0.5	>32	>32
ERG11^{AA}	NA	NA	NA	E266D G307S G450E V488I	A61E E266D G307S G450E V488I	NA	A61E E266D G307S G450E V488I	A61E E266D G307S G450E V488I	A61E E266D G307S G450E V488I
ERG11(-)^{GX}	NA	NA	NA	4.85	12.3	NA	0.43	5.70	3.44
CDR1(-)^{GX}	NA	NA	NA	1.69	7.40	NA	2.95	4.73	1.45
CDR2(-)^{GX}	NA	NA	NA	69.2	868.1	NA	194.8	132.5	14.5
TAC1^{AA}**	NA	NA	NA	R688Q^{h}	R673L	NA	R673L	R673L	R673L
FKS1^{AA}	NA	NA	NA	V661F^{h}	V661F^{h}	NA	S645P^{h} V661F^{h}	S645P^{h} V661F^{h}	S645P^{h} V661F^{h}
ERG2^{AA}	NA	NA	NA	WT	WT	NA	F105fs^{h} ***	F105fs** *	F105fs* **

7.2EUCAST (E.def 7.2) MIC values (mg/L), ETEtest (mg/L), AAAmino acid changes, GXRelative gene expression, NA: Not available, hheterozygous, PPrimary specimen, CCulture.

FLU: fluconazole, ITZ: itraconazole, VRZ, voriconazole, POS, posaconazole. ANI: anidulafungin, CAS: caspofungin, AMB: amphotericin B.

WT: wild-type susceptibility, F: Fluconazole resistant, A: azole resistant, E: echinocandin resistant, MDR, multidrug resistant.

MIC values above clinical breakpoints and regarded as resistant are highlighted grey. Underlined amino acid changes are known to be associated with resistance.

**Trailing phenotype with approximately 50% growth inhibition in the concentration range 0.5-4 mg/L.*
***The TAC1 gene sequence harboured multiple non-synonymous mutations but only potential GOF mutations are shown.*
****Frameshift mutation F105SfsX23 due to basepair deletion (T314).*

Two amino acid changes (G307S and G450E) in *ERG11*, found in isolate P-4, were probably the significant drivers of the observed fluconazole resistance [90] and may have been further potentiated by elevated expression levels of *ERG11* and particularly *CDR2*. Pan-azole resistance, observed in P-5, was likely inflicted by the additional A61E amino acid change in *ERG11* and upregulated expression of *ERG11*, *CDR1* and *CDR2*. The position of A61E in *ERG11* was modelled to understand the role of this novel variant in relation to itraconazole affinity (Figure 12). Indeed, a potential steric interference between the polar side chain of glutamic acid and itraconazole binding was observed, and could be a plausible explanation for reduced susceptibility to long tailed triazoles (itraconazole and posaconazole).

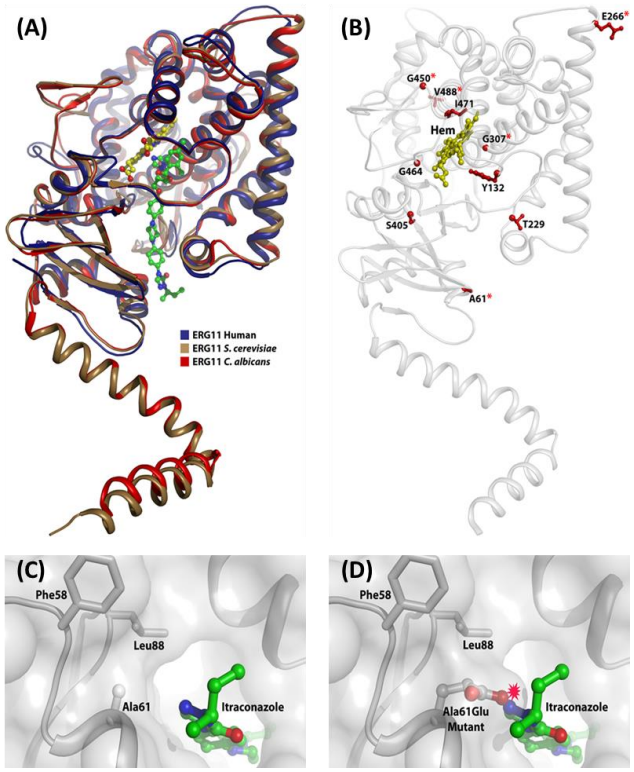


Figure 12. *C. albicans ERG11* protein homology modelling. (A) Superposition of *ERG11* crystal structures from Human and S. cerevisiae and the Phyre2 model [124] of *C. albicans*. (B) Position of amino acids, which have been found altered in azole resistant *C. albicans* [83]. Red asterisks indicate the AA site, which was altered in the pan-azole resistant isolates in Paper III. (C) Close-up of the Phyre2 model of *ERG11* of *C. albicans* with Itraconazole superimposed into the binding site. (D) Position of the Ala61Glu (A61E) mutation in *C. albicans*, which potentially interferes with the tail of itraconazole and being responsible for resistance to long-tailed azoles.

The location of other relevant amino acid sites were also shown including G307 and G450. Still, the presented model can only provide indications of the actual molecular and structural events of such changes and may merely be used for visual understanding and theoretical support in relation to azole resistance [94, 95]. The observed gene expression for *ERG11* was not coupled to mutations in *UPC2* and thus a compensatory mechanism, for the potentially reduced catalytic activity of *ERG11*, leading to *ERG11* upregulation deserves further investigation. *CDR1* and *CDR2* on the other hand, were potentially induced by *TAC1* due to a supposed novel GOF variant R673L. Moreover, *TAC1* had underwent a major LOH event rendering the entire gene homozygous in P-5 to P-9 as opposed to P-4 and the circumstances of this event would be interesting to study further [125].

Echinocandin resistance was induced by the acquisition of a well-known S645P substitution in *FKS1* due to heterozygous mutations in P-7 through P-9. Finally, amphotericin B resistance was probably linked to a frameshift mutation in *ERG2* conferring a severely truncated protein structure (from 217 to 126 AAs). In support of this hypothesis, we showed ergosterol depletion in the amphotericin B resistant isolates (P-8 and P-9) and found sterol profiles, which were similar to what was observed in other *Candida* species displaying *ERG2* associated AMB resistance [117, 126]. Additional sterol profiles for the isogenic isolates have been presented in supplementary reading (S.3 Additional sterol profiles of *ERG11* mutants from Paper III) and indicated that the mutations in *ERG11* may led to a lowered catalytic activity of *ERG11* and thus a reduced the ergosterol biosynthesis.

Reduced fitness in resistant strains is a well-known phenomenon. GOF mutations in *TAC1* and *UPC2* are previously shown to attenuate virulence [122] and ergosterol deplete *C. albicans* were unable to form pseudohyphae and had delayed growth and reduced virulence [127]. Resistance to echinocandins is shown to be associated with cell-wall instability and especially the S645P variant has been shown to confer reduced catalytic capacity of *FKS1* leading to increased cell wall chitin content, which in turn attenuated fitness and virulence [50, 72, 75, 128]. We studied virulence in our resistant strains in the insect model Galleria mellonella caterpillars [129]. Besides the isogenic and increasingly resistant strains, two unrelated wild-type control strains (C-1 and C-2) and one control strain resistant to azoles and echinocandins (C-3) were included (Figure 13).

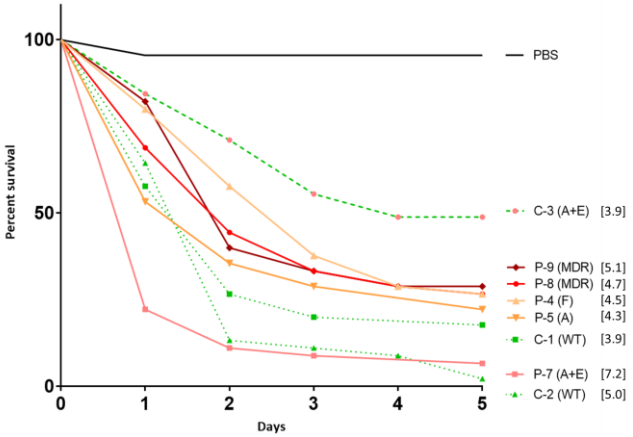


Figure 13. Virulence in the Galleria mellonella larvae model. Letters in parenthesis denote susceptibility profiles: WT, wild-type susceptibility; F, fluconazole resistant; A, azole resistant; and E, echinocandin resistant. Mean cells/larva injected (×105) are indicated in square brackets. Broken lines indicate reference strains and solid lines indicate clinical isolates (reproduced with permission from the publisher Oxford University Press).

Expectedly, the isogenic and increasingly resistant strains were less virulent (also taking the CFU variation into account). Interestingly, however, the azole and echinocandin resistant control strain, C-3, displayed a more pronounced loss of virulence. Still, the genetic background in C-3 was different than the clinical strains, thus whether virulence cost was truly abrogated by compensatory mechanisms in the clinical strains is unclear. Still, these results showed an only slightly reduced virulence, which may have played a role in the long-term persistence in the patient, potentially transcending from the oesophagus (P-1 to P-6) and through to the colon (P-7 to P-9). Potential sub-therapeutic drug concentrations in the oesophagus and the extensive treatment course may have enabled the development of unknown compensatory mechanisms, mediating a somewhat regained level of virulence.

This study was possibly the first to cover resistance against all three drug-classes in *C. albicans*, whereas multidrug resistance in *C. glabrata* have been reported previously although with a less degree of genetic support [79, 130]. We proposed several novel resistance mechanisms and they should ultimately be further investigated. Whole genome sequencing of these strains could indeed help resolve the true genetic landscape responsible for the rare phenotypic MDR trait. One next-generation sequencing strategy have recently been presented, assessing echinocandin and azole resistance in 40 *Candida* isolates by mapping six genes (*ERG11*, *ERG3*, *FKS1*, *FKS2*, *TAC1* and PDR1) often involved in resistance [131]. Besides presenting known as well as potentially novel resistance mutations, this study illustrated the future potential of deep sequencing methods for the understanding of antifungal resistance mechanisms in *Candida*.

2.6 Is resistance underestimated in fungaemia programmes? (Paper IV)

Several cases of acquired resistance were presented here and may indicate an increasing prevalence in Denmark. One question arises, whether we overlook something basing our estimates on the fungaemia programme, where acquired resistance remains rare [18]. One reason for this concern is that fungaemia programmes only involve the first blood culture isolate (where the patient ultimately has been least exposed to antifungals) and not subsequent isolates unless separated by more than 3-4 weeks (depending on the scheme). Thus, as suggested previously [18, 31], we might only see the tip of the iceberg and underestimate the occurrence of acquired resistance as potentially subsequent resistant isolates were never captured. Few studies have evaluated prophylactic treatment in correlation with subsequent *Candida*emia [132, 133]. Both studies demonstrated a significantly altered species distribution among *Candida*emia in patients previously exposed to either fluconazole or echinocandins towards species intrinsically less susceptible to either drug class. Indeed, increased prevalence of intrinsically resistant species is a prominent clinical concern. To address the effect of antifungal exposure in *Candida*emia patients, we undertook a study (Paper IV) [134], where post-treatment mucosal isolates were obtained (Figure 14). Project material and required approvals are provided in supplementary reading (S.4 Supplementary material for Paper IV).

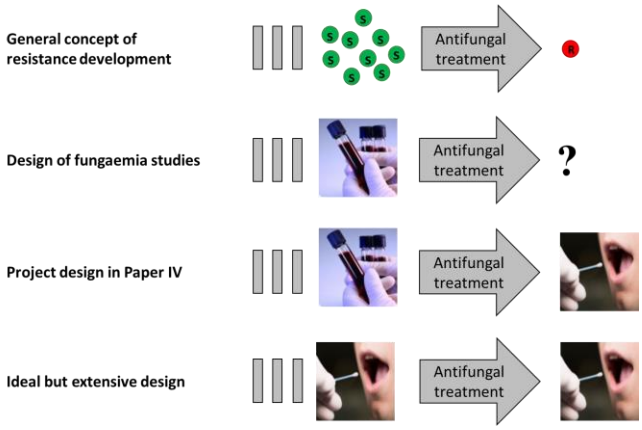


Figure 14. Project design in Paper IV. Upon antifungal treatment *Candida* isolates may develop resistance to a larger extent than reported in fungaemia studies. This could be elucidated by the implementation of follow-up samples from *Candida*emia patients post treatment, which is what we described in Paper IV, pairing oral isolates with initial blood isolates, which were already routinely referred. Ideally, an initial oral swab should have been obtained but such study design would have been logistically too extensive not to mention the numerous additional isolates requiring analyses.

The design of this study was a systematic multicentre study where oral swabs were collected from 193 *Candida*emia patients after antifungal exposure. Two questions were investigated for patients exposed to either azoles (N=114) or echinocandins (N=85) (some patients received both):

1) What influence did antifungal exposure have on the species distribution in colonising *Candida*?
2) What was the extent of acquired resistance in colonising *Candida* upon antifungal exposure?

Since all *Candida*emia patients were treated with an antifungal and because initial oral isolates were not obtained due to logistical constraints, an unexposed control group lacked in question 1. Instead, blood isolates were regarded as controls and species distributions were thus compared between blood isolates and oral isolates from patients exposed to either azoles or echinocandins (Figure 15). The premise for such approach was that the infectious agent and the concomitant colonizing *Candida* species have been shown to be genetically identical in more than 90% of cases and that most patients were permanently colonised independently of infection [32, 135–140]. The 90% correlation between initial blood isolates and subsequent paired oral isolates was also what we showed through genotyping for those species, where an established typing scheme was adopted [73, 120, 141–145].

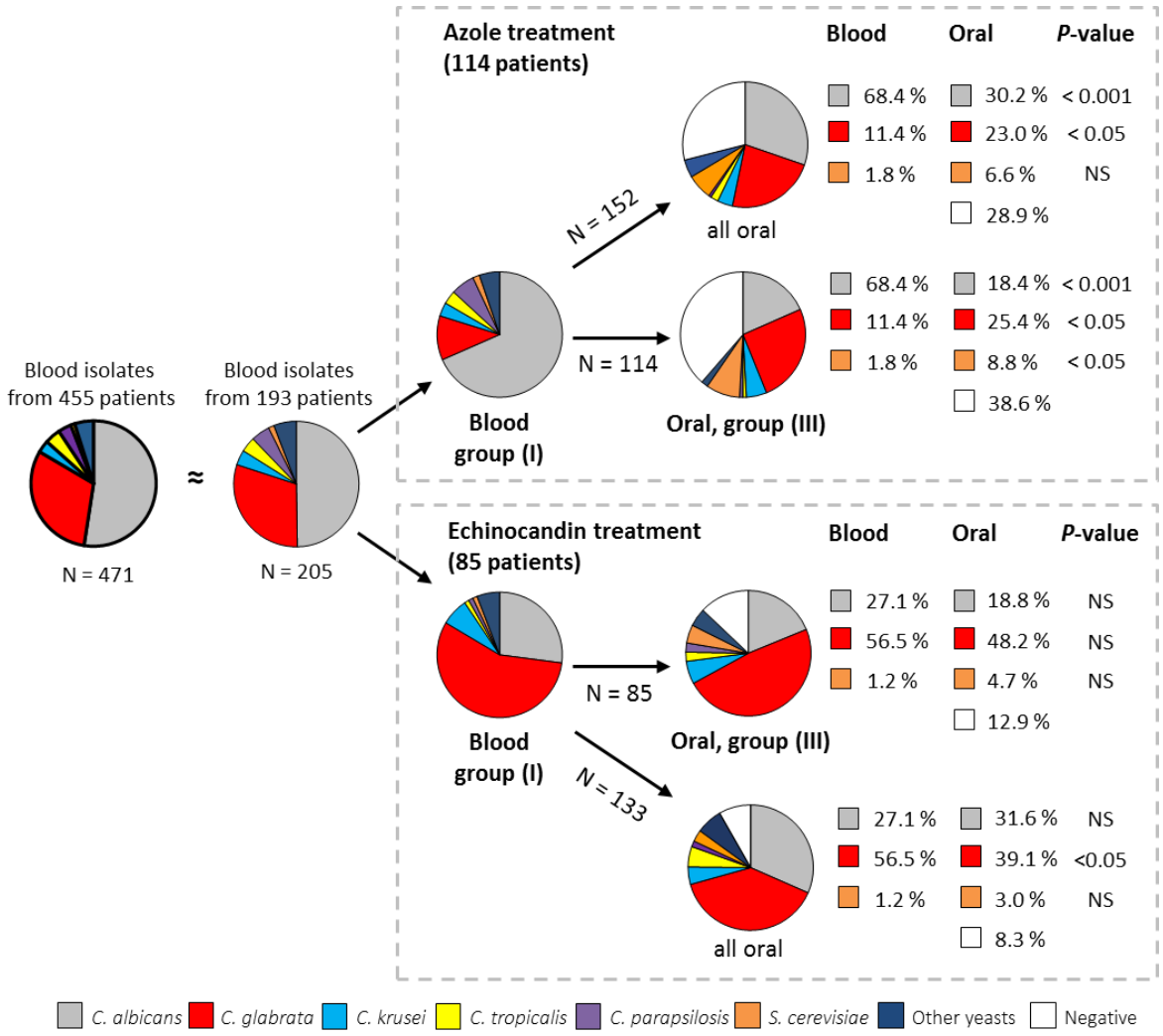


Figure 15. Species distributions among blood and oral isolates. Pie-charts displaying species distributions in the indicated groups. N, Number of isolates. Blood isolates, group (I) represented baseline colonization in patients exposed to either azoles or echinocandins. Group (III) oral isolates (≥7 days exposure) represented end of treatment colonization. NS, not significant. On patient level (horizontal), only the species with the highest ECOFF was counted in case of polyfungal samples but the distribution of all oral isolates have also been shown (above for azoles and below for echinocandins). Proportion analysis was performed by chi-squared or Fisher's exact tests and P-values <0.05 were considered significant. No differences were observed for *C. krusei*, C. *tropicalis*, C. parapsilosis and other yeasts in neither treatment arm and P-values were thus not presented.

Importantly, we demonstrated significant differences in species distributions among blood and oral isolates in azole exposed patients, most prominently for *C. albicans* and *C. glabrata*, but not in echinocandin exposed patients (detailed data is provided in supplementary reading, S.4 Supplementary material for Paper IV). One interesting finding was the proportion of culture negative oral isolates being significantly lower in azole exposed patients compared to echinocandin exposed patients. One hypothesis was that azoles clear the colonising *Candida* on mucosal surfaces more effectively than echinocandins. This could partly be explained by the substantiated lower protein binding and associated higher drug concentrations at the mucosal surfaces. Still, because pretreatment oral swabs was not obtained, we cannot rule out that more patients in the azole group, theoretically might have been swab culture negative before treatment, thus ruling out the subsequent effect of exposure. This would on the other hand not explain the significantly higher prevalence of polyfungal oral samples among the echinocandin treated patients, which was also observed.

Table 7. Resistance proportions among *Candida glabrata* blood and oral isolates.

	Isolates (exposure to azoles)		
Comparisons	**Oral (≥7 days)***	**Blood (no exposure)**	**Oral (<7 days)**
Fluconazole MIC above BP, no. of isolates/total (%)	10/34 (29.4%)	3/62 (4.8%)$^{<0.01}$	5/48 (10.4%)$^{<0.05}$
Fluconazole geometric mean MIC (mg/L)	10.01	3.66$^{<0.05}$	4.83$^{<0.05}$

	Isolates (exposure to echinocandins)		
	Oral (≥7 days)*	**Comparisons**	**Oral (≥7 days)***
Anidulafungin MIC above BP, no. of isolates/total (%)	11/51 (21.6%)	3/62 (4.8%)$^{<0.01}$	1/31 (3.2%)$^{<0.05}$
Anidulafungin geometric mean MIC (mg/L)	0.053	0.043^{NS}	0.048^{NS}

BP: EUCAST clinical breakpoint for resistance.
**Reference column for statistical comparisons. Exposed ≥7 days to an azole or an echinocandin before the oral swab was obtained. Controls were either blood isolates or oral isolates from patients exposed <7 days to either antifungal.*
Superscript numbers indicate significant P values, NS: not significant.

The observed rates of acquired resistance in *C. glabrata* to fluconazole and echinocandin resistance were much higher than those presented in the recent surveillance studies [31, 146]. This further emphasised the potential underestimation of resistance in *Candida* species. High resistance rates have been presented previously both for echinocandins [19, 147, 148] and azoles [133, 136, 148] and again the site of infection seemed to play a role. Thus, it is hypothesised that the oral fungal microbiota may be an unrecognised reservoir of resistant *Candida* species (especially *C. glabrata*) in *Candida*emia patients following treatment. Furthermore, acquired azole and echinocandin resistance in *C. glabrata* was common and add to the concern that this organism may become an important "multidrug resistant" yeast challenge of our time [149–152].

2.7 Bridging Candida and Aspergillus

Despite that *Candida*emia is considered among the top five of the most prevalent nosocomial bloodstream infections (depending on the patient population), only rare cases of hospital outbreaks have been reported and primarily with C. parapsilosis in paediatric settings [153–156]. Thus, despite the recognised understanding that invasive candidiasis is related to the concomitant colonising *Candida* it remains unclear to what extent the colonising fungal microbiota is influenced by exogenous *Candida* [138, 139]. Only a few studies have investigated the potential concern of an exogenous source and presented the occurrence of resistant *Candida* on fruit and vegetables from (conventional) orchards displaying cross-resistance to clinical azoles [157, 158]. Although, this may be a negligible concern since the primary cause of invasive candidiasis is from a constant colonising microflora [139, 159] it would be interesting to pursue. In contrast, the current situation of resistant mould infections caused by the spore-producing airborne *Aspergillus fumigatus* potentially originating from the environment is now a worldwide concern [2].
For question 2, the number of isolates with MICs above the breakpoints was again compared between blood and oral isolates but an additional control group was defined. Oral isolates from patients exposed to <7 days of azoles or <7 days of echinocandins were applied as appropriate controls when assessing azole and echinocandin resistance respectively (Table 7).

PART III: RESISTANCE IN THE UBIQUITOUS MOULD *ASPERGILLUS FUMIGATUS*

3.1 Aspergillus fumigatus causes severe pulmonary infections

Among the spore producing *Ascomycetes* causing invasive infections, *Aspergillus* is the most prevalent genus represented primarily by *A. fumigatus* and less frequently *Aspergillus* terreus, *Aspergillus* nidulans, *Aspergillus* niger and *Aspergillus* flavus [160]. The properties of the asexually produced spores makes this organism an airborne concern to human health. The fact that an average person inhales hundreds of spores daily may help explain the wide range of pulmonary diseases inflicted by *Aspergillus* species [161]. This covers allergic bronchopulmonary aspergillosis, chronic respiratory diseases and severe invasive infections [162, 163]. On a global measure, *Aspergillus* is estimated to cause health issues in millions of people annually, with invasive aspergillosis (IA) accounting for approximately 200,000 annually [162, 163]. In Denmark, chronic *Aspergillus* diseases and infections may be relatively frequent especially among cystic fibrosis (CF) patients while IA is rare and estimated to 50-60 cases/annually (or 0.9-1.1/100,000 inhabitants) [27]. Neutropenic patients lack neutrophils, which are an essential part of the innate immune defence against microbial infections, and thus such patients are highly prone to acquiring IA [164]. Further, challenges for those infections are the limited therapeutic options associated with acceptable response rates but also the difficulties in performing a correct diagnosis in time. The subgroup of the European Organization for Research and Treatment of Cancer (EORTC), Mycoses Study Group (MSG) published revised definitions for the diagnosis of invasive aspergillosis for clinical studies, which are divided in different significance levels; proven, probable and possible infection depending on the degree of evidence [165]. These definitions illustrate the complexity of establishing the diagnosis and that further diagnostic tools are needed to improve the prognosis of patients with IA. Indeed, early diagnosis as well as severity of underlying diseases impact the mortality rate of IA, which is acknowledged to be in the range of 30-50% [166, 167] but those numbers are alarmingly high (>80%) when the causative agent is resistant [163]. Since azoles constitute first line therapy of most *Aspergillus* infections, azole resistance is unquestionably the most significant clinical concern with respect to the management of aspergillosis.

3.2 Aspergillus and azole resistance – an emerging threat (Paper V-VI)

The increasing number of international reports addressing azole resistance in *A. fumigatus* reflects the worldwide focus on this emerging threat. While the variety of resistance mechanisms may be equally complex as for *Candida* species, about 90% of azole resistance cases in *A. fumigatus* has thus far, been linked to genetic changes of *CYP51A* (corresponding to *ERG11* in *Candida*) [168]. Consequently, structural changes of the azole target protein as well as upregulation is responsible for the observed resistance. The other 10% remain primarily unresolved, although increased drug efflux [169] and a potential GOF variant in a transcription factor complex subunit HapE have also been characterised as drivers of azole resistance [170]. In recent years, there has been an extraordinary focus on azole resistant *A. fumigatus* iso-

lates potentially originating from the environment, and displaying cross-resistance to clinical azoles [2]. In fact, resistant *A. fumigatus*, supposedly derived from the environment, is increasingly found both in clinical and environmental samples [171]. The actual threat was emphasized by the discovery of genetically related azole-resistant *A. fumigatus* isolates found in azole naïve patients and the surrounding environmental samples [172–174]. The fact that azole resistance rates are increasing, especially in the Netherlands where almost 90% of clinical azole resistant *A. fumigatus* is carrying environmentally derived resistance mechanisms further substantiates the overall concern [171]. The dominant environmental azole resistance mechanisms are two similar genetic signature variants (Figure 16). The most prevalent is a 34-basepair tandem repeat in the promotor region combined with a mutation in the target gene leading to the amino acid substitution L98H, thus dubbed TR_{34}/L98H, conferring pan-azole resistance [171]. Later, a 46-basepair tandem repeat in the promotor region combined with two non-synonymous mutations in *CYP51A*, denoted TR_{46}/Y121F/T289A, conferring high voriconazole resistance, was described [174].

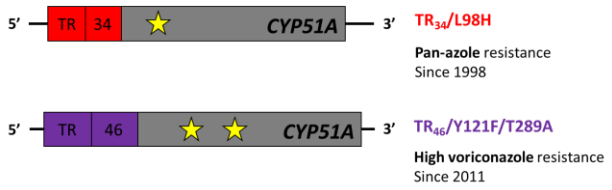


Figure 16. Two environmentally derived resistance mechanisms. Simplified illustration of the TR_{34}/L98H (above) and TR_{46}/Y121F/T289A (below) resistance mechanisms. Stars indicate mutations in *CYP51A* conferring AA substitutions. TR is a tandem repeat in the promotor region. Further detail on the tandem repeats can be found in supplementary reading (S.5 Sequence profiles of *CYP51A* promotor regions).

3.2.1 Azole resistance acquired ex vivo originating from the environment

The European Centre for Disease Control (ECDC) published a risk assessment of the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species [175]. The group of experts coordinated by ECDC presented the extent of this problem and also summarised evidence for the environmental origin of azole resistance development. The arguments are:

1) Azole resistant *A. fumigatus* isolates have been recovered from patients with no history of previous azole exposure (azole naïve patients) [176].
2) Azole resistant *A. fumigatus* isolates have been recovered from environmental samples and, almost exclusively, the two variants TR_{34}/L98H or TR_{46}/Y121F/T289A [177].
3) In the Netherlands and other countries, the two mechanisms TR_{34}/L98H and TR_{46}/Y121F/T289A are stable and dominating mechanisms among clinical and environmental isolates. In other populations including a high proportion of azole treated patients with chronic forms of aspergillosis, as in the UK, a more heterogeneous population of *CYP51A* variants exists. It is assumed that the in vivo selection of resistance may result in a more diverse panel of resistance mutations in *CYP51A* [168].
4) The two "environmental" resistance mechanisms possesses two independent genetic events (mutations and a tandem repeat), which has not been found previously in any case of in vivo resistance development but present in azole resistant plant pathogenic moulds [2, 178].
5) Environmental TR_{34}/L98H isolates cluster genetically to clinical TR_{34}/L98H isolates but distinct from any other susceptible wild-type isolate [179, 180].
6) The TR_{34}/L98H isolates are cross-resistant to several triazole fungicides which were introduced in the agriculture just few years before the first detection of a TR_{34}/L98H isolate in 1998 (tebuconazole, propiconazole, difenoconazole, epoxiconazole and bromucazole) [181, 182].
7) These triazole fungicides display similar structure as clinical azoles (thus, they theoretically possess same selection potential) (Figure 17). Moreover, they have been shown to induce tandem repeats in *CYP51A* in vitro, conferring reduced susceptibility to clinical azoles [181].

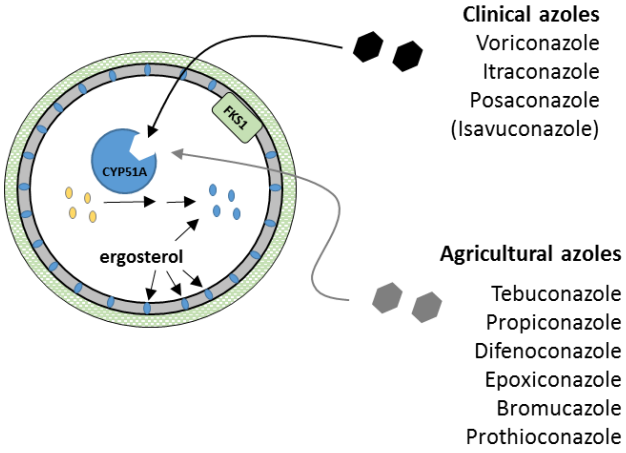


Figure 17. *CYP51A* inhibition by clinical and agricultural azoles. Simplified illustration of clinical and agricultural azoles being structurally similar. Tebuconazole has been shown to induce tandem repeats in the *CYP51A* promotor region in laboratory experiments [181].

In relation to the third argument by ECDC, it has been argued that the resistance mechanism TR_{34}/L98H in *A. fumigatus* was not associated with a fitness cost [181]. This supported the possible emergence of a stable clone able to proliferate equally among wild-types even in the absence of azole selection (confer scenario IV in Figure 3). While the TR_{34}/L98H resistant clone emerged in a Dutch patient in 1998 we described the first TR_{34}/L98H case in Denmark in a CF patient (from 2007) in 2011 [183]. Equivalently, the TR_{46}/Y121F/T289A was found in a Dutch patient in 2009 and we subsequently described the first TR_{46}/Y121F/T289A case in Denmark along with three additional TR_{34}/L98H cases in 2014 in Paper V [184]. All four cases had a lethal outcome, underlining the severity and poor prognosis of such resistant infections. In correlation with the first argument by ECDC, two of the patients were azole naïve at the time, the resistant isolates were discovered. This indicated that the patients acquired the resistant isolates by inhalation in the environment. Case 2 was co-infected with a Zygomycetes mould, which may have delayed the IA diagnosis. Furthermore, three patients were co-infected with genetically distinct wild-type susceptible *A. fumigatus*, one of which (Case 1) may have been undetected if STRAf genotyping had not resolved the presence of mixed isolates (case overview presented in Table 9).

Due to the continuous findings of the environmental resistant genotypes among clinical isolates, and now also outside the CF population, we investigated the occurrence in the environment in

two additional surveys (Table 8). Surprisingly, no resistant isolates were discovered in the environment in spite of a larger sample material compared to the study from 2010 [185]. Still, seasonal and climate variations were suggested to play a role compared to equivalent surveys in other countries. Indeed, the recent surveys were carried out either in early fall or early spring and only in countries with high prevalence or a warmer climate, environmental resistance has been detected throughout the year [184].

Table 8. Environmental samples from Paper V. Soil samples and *A. fumigatus* findings from the two environmental surveys (2010 and 2013) compared with the data from a previously published study performed in 2009 (reprinted from [184] with permission by the publisher, ASM).

					Farms			
	Tivoli		Hospital		Conventional		Organic	
	2009*	2010	2009*	2010****	2010	2013	2010	2013
No. soil samples (%)	23 (100)	17 (100)	27 (100)	25 (100)	12 (100)	130(100)	15 (100)	40(100)
No. *A. fumigatus* (%)	21 (91)	15 (88)	17 (63)	19 (76)	11 (92)	45 (35)	13 (87)	10 (25)
No. resistant (%)	3 (13)***	0	1 (4)**	0	0	0	0	0

**Data compiled from previous study [185].*
***Or 6% of A. fumigatus isolates*
****Or 14% of A. fumigatus isolates*
*****Indoor*

Table 9. *A. fumigatus* case overview from Paper V. Mould isolates, diagnostic delay, MICs, resistance genotypes (*CYP51A* profiles) and STRAf typing of clinical isolates obtained from the four patients (reprinted from [184] with permission by the publisher, ASM).

					MIC (mg/L)				STR*Af*	
CASE	Day	Site*	Days delay**	Species	POS	VRZ	ITZ	*CYP51A* profiles	(2A-2B-2C-3A-3B-3C-4A-4B-4C)	Out-come
1	7	BAL	12/11	*A. fumigatus*	0.06	1	0.25	WT	18-19-8-26-10-21-9-9-5	Died
	7	BAL	12/11	*A. fumigatus*	1	4	>8	TR_{34}/L98H/S297T/F495I	14-10-9-30-9-6-8-10-20	
	17	BAL	14/7	*A. fumigatus*	0.5	1	>8	TR_{34}/L98H/S297T/F495I	14-10-9-30-9-6-8-10-20	
2	44	BAL	18/8	*A. fumigatus*	0.03	0.25	0.125	WT	14-20-11-34-9-7-8-10-12	Died
	90	TS	20 /18	*A. fumigatus*	0.5	4	>8	TR_{34}/L98H	25-10-12-79-9-9-8-10-11	
	90	TS	20/18	*R. pusillus*	0.25	>4	0.5	NA	NA	
	106	TS	13/9	*R. pusillus*	0.125	>4	0.25	NA	NA	
	110	TS	9/8	*R. pusillus*	0.125	>4	0.25	NA	NA	
	117	TS	9/8	*A. fumigatus*	≤0.03	0.5	0.25	WT	25-16-19-48-17-23-8-9-5	
	117	TS	9/8	*R. pusillus*	0.25	>4	0.25	NA	NA	
3	6	BAL	16/ 10	*A. fumigatus*	0.5	4	>8	TR_{34}/L98H	20-20-28-32-9-6-8-10-20	Died
4	-7	BAL	26/11	*A. fumigatus*	0.06	0.5	0.125	WT	18-25-15-26-11-7-26-30-8	Died
	36	Sputum	7/6	*A. fumigatus*	0.125	>4	0.25	TR_{46}/Y121F/T289A	26-21-16-32-9-10-8-14-10	
	58	Sputum	ND/7	*A. fumigatus*	0.25	>4	0.5	TR_{46}/Y121F/T289A	26-21-16-32-9-10-8-14-10	
	62	Sputum	10/9	*A. fumigatus*	0.25	>4	0.5	TR_{46}/Y121F/T289A	26-21-16-32-9-10-8-14-10	

NA: Not applicable, ND: not determined, CYP51A and STRAf genotyping was only applicable to A. fumigatus. WT, wild-type; POS, posaconazole; VRZ, voriconazole; ITZ, itraconazole.
**Origin of sample: BAL: bronchoalveolar lavage. TS: tracheal sputum/aspiration.*
***Diagnostic delay (from initial sampling to microbiological diagnosis/from sample arrival at reference laboratory to microbiological diagnosis).*

3.2.2 The worldwide extent of azole resistant A. fumigatus derived from the environment

The extent of azole resistant *A. fumigatus* carrying environmentally derived azole resistance is now worldwide (Figure 18) with the recent findings TR_{34}/L98H and TR_{46}/Y121F/T289A isolates all over Europe [171, 174, 179, 180, 183, 184, 186–205], in North America [206, 207], Colombia [208], India [209], China [201] and Tanzania [190].

Besides the lack of studies addressing this concern in many parts of the world, another important point may explain the high occurrence mainly in Europe and Asia compared to other parts of the world. Triazole fungicide consumption was assessed in a recent study [182] and the authors found that out of the overall world consumption, western Europe and Asia-Pacific accounted for 37% and 24% respectively, thus almost two thirds. Besides the unknown travel-patterns of *A. fumigatus* within air, compost, flower pots etc., triazoles fungicide use could indeed be a valid explanation for this current relative confinement of azole resistant *A. fumigatus* [182].

Besides the two dominating resistance mechanisms above, other resistance mechanisms (G432S and TR_{53}/WT) found in isolates from azole naïve patients were suggested to be acquired from the environment [212, 213] and additionally environmental surveys have identified two AA variants, most often involved in in vivo resistance development (M220I and G54A)[203].

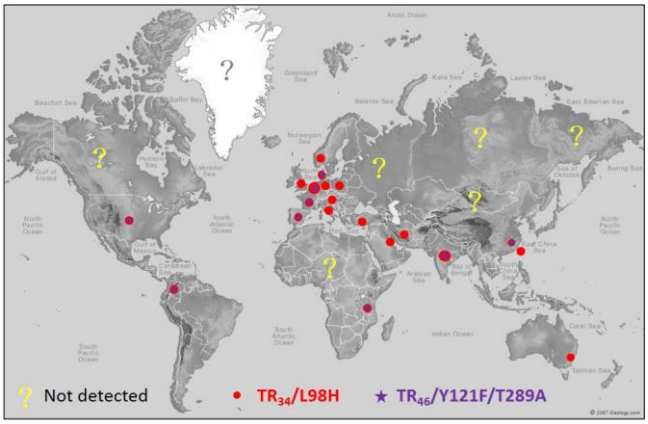


Figure 18. Geographical distribution of TR_{34}/L98H and TR_{46}/Y121F/T289A isolates. There are still many countries in the world were these resistant clones have not been discovered but for the most part, it may merely be a matter of the lack of sampling rather than absence.

3.2.3 In vivo acquired resistance upon azole treatment

Genotyping has allowed tracing of initially susceptible isolates with resistant progeny selected upon azole exposure both in vitro and in vivo harboring mutations in *CYP51A* [214, 215]. The list of known mutations conferring AA substitutions associated with resistance was recently reviewed [182] and an updated Table 10 is presented below.

As for *C. albicans* the *CYP51A* protein in *A. fumigatus* has been modelled and significant amino acid sites were identified in relation to the heme center to substantiate the importance for azole access and potentially resistance (Figure 19) [221, 222]. Genotyping also assisted in the detection of in vivo selection of azole resistance mutations in *CYP51A*, developed during azole exposure (M220I and P216L) in [183] and Paper VI [223]. We also demonstrated an equivalent *CYP51A* variant in A. terreus (M217I, corresponding to M220I in *A. fumigatus*) acquiring azole resistance upon azole exposure [224].

Table 10. *CYP51A* amino acid substitutions associated with azole resistance [182]. This list comprises amino acid variants, which have been found in clinical and/or environmental isolates displaying reduced azole susceptibility patterns as indicated. The AA substitutions were found as sole variations unless indicated otherwise.

CYP51A	**Association with resistance**			
AA substitution	**ITZ**	**POS**	**VRZ**	**Comment**
WT	S	S	S	The wild-type variant
N22D	R	NA	NA	
G54x	R	S/I/R	S	E, K, R, V, W variants. G54A in environment [203]
L98H	R	S/I/R	I/R	Found with TR_{34} and engineered in [216]
Y121F	S	S	R	Found alone [217] and high VRZ resistant with TR_{46}/T289A
G138x	S/R	S/R	R	C, R variants
H147Y	R	I	R	
P216L	R	I/R	S/I	
F219x	R	S/I/R	S/I/R	I, S variants. M220I also in environment [203]
M220x	R	S/I/R	S/I/R	K, I, T, V variants
I242V	I/R	NA	NA	[206]
I266N	R	I	S	Probably N266I [218]
A284T	S/I	S/I	S/I	
T289A	S	S	S	Engineered [219] and high VRZ resistant with TR_{46}/Y121F
TR_{34}/L98H	R	S/I/R	I/R	
TR_{46}/Y121F/T289A	I/R	I/R	R	
S297T	S	S	S	Often found with TR_{34}/L98H/F495I
F332K	R	I	S	Probably P332K [182]
S400I	S	S	I	
E427G	R	S/I	I/R	
Y431C	I/R	S/R	S/I/R	
G432S	R	S	S	Also found in azole naïve patient [212]
G434C	R	R	R	
T440A	R	NA	NA	
G448S	R	I/R	R	Also described in [220]
Y491H	R	NA	NA	
F495I	S	S	S	Often found with TR_{34}/L98H/S297T

S, susceptible, I, intermediate, R, resistant according to EUCAST breakpoint definitions [21] and confer Table 1.

The clinical implication was in this case potentiated by the intrinsic polyene resistance in A. terreus, but fortunately acquired azole resistance in A. terreus has to our knowledge remained a very rare event. Overall, such cases illustrate the constant concern for patients undergoing long-term azole exposure. On a second note, mutants suspected to derive in vivo display higher diversity both with regards to resistance mutations in *CYP51A* but also with regards to STRAf genotyping profiles as observed previously [180, 198].

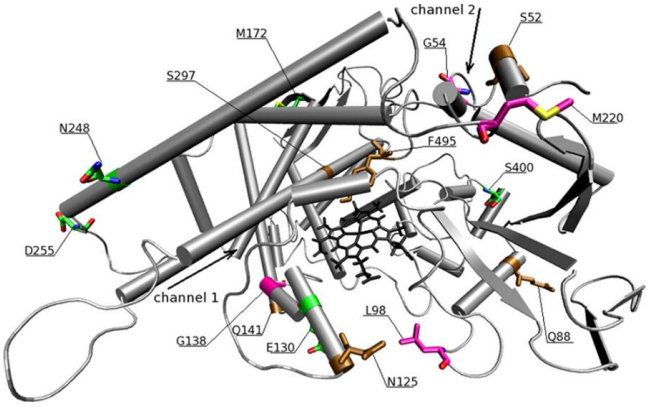


Figure 19. *CYP51A* protein model of *A. fumigatus*. The model includes important amino acids relevant in association with azole susceptibility and most amino acids have been experimentally validated as drivers of resistance. The illustration is printed from a previous paper [221] with permission from the publisher ASM.

3.2.4 Prevalence of azole resistant A. fumigatus in Denmark

In Denmark, four studies have addressed azole resistance in *A. fumigatus* both from environment and clinical isolates [27, 183–185] describing a prevalence of 8% in soil samples in the first environmental survey but 0% in two subsequent environmental studies. One study prospectively investigated azole resistance in clinical isolates during 3 months. This study presented an azole resistance prevalence of 4% of which two isolates were *A. fumigatus* and only one harboured a known *CYP51A* variant (M220K) associated with azole resistance. Moreover, a Danish CF cohort was sampled in which a prevalence of 4.5% azole resistant *A. fumigatus* isolates were found in 2007 and 2009 [183]. This cohort probably represented a population where acquired resistance is higher than in the entire Danish population because CF patients often undergo long-term antifungal exposure due to their inherited predisposition for *Aspergillus* disease. Since Paper V showed that environmentally derived azole resistant *A. fumigatus* persist in Danish clinical samples [184], there has been an increasing demand to uncover the prevalence of azole resistance.

An assessment of the susceptibility data of different *CYP51A* variants encountered among Danish *A. fumigatus* isolates is provided in supplementary reading (S.6 EUCAST susceptibility of Danish *A. fumigatus* isolates with *CYP51A* mutations).

Paper VI [223] was a laboratory based retrospective study from 2010-2014, which investigated the occurrence of azole resistant *A. fumigatus* as well as the underlying resistance mechanisms and genotyping data. An increasing number of *A. fumigatus* isolates were obtained, either as referred isolates or as culture positive of primary specimens, and the proportion of susceptibility tested isolates also increased (Figure 20). Moreover, an increase in azole resistance was seen both with regards to isolates, to 6% in 2014 ($P < 0.001$) and patients, to almost 4% in 2014 ($P < 0.05$) (Figure 20).

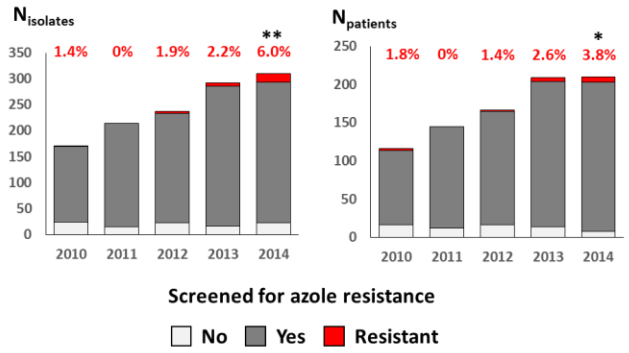
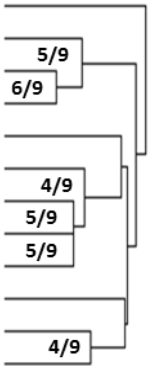


Figure 20. Referred *A. fumigatus* isolates from 2010-2014. Overview of *A. fumigatus* isolates (left) and corresponding patients (right) examined at Statens Serum Institut from 2010-2014. Grey indicates when the isolates were screened for azole resistance, white indicates isolates not tested and red indicates resistant isolates. Red numbers above each bar indicate percentage of resistance (among susceptibility tested isolates).
*P < 0.05 **P < 0.001, chi-squared test for trends in proportions.

3.2.5 Strengths and pitfalls of A. fumigatus genotyping, what have we learned?

During the study period for Paper VI there was a potential outbreak at a haematology unit (2012-2013). This was assessed by analysis of an increased number of patient specimens (*Aspergillus* galactomannan antigen, culture, microscopy, susceptibility patterns and genetic analysis if culture positive) as well as samples from seven ventilation filters. In short, the outbreak was not resolved and a single common source of *A. fumigatus* was undetected (Table 11). However, since 13 of 14 patients had positive biomarker (galactomannan antigen) results within a short period of time and because the ceiling in connecting hall-ways were undergoing repair, an outbreak associated with the construction work was indeed a plausible explanation for this increased incidence.

Table 11. The Roskilde *A. fumigatus* outbreak. Microsatellite typing, susceptibility data and demographics.

	Acquired		Source	Clinical Outcome	EUCAST MIC (mg/L)			STR*Af* microsatellite typing									Comparison
Patient*	Date	Site	City		ITZ	POS	VRZ	2A	2B	2C	3A	3B	3C	4A	4B	4C	Dendrogram
SSI-19[PBL]	04-06-13	TS	Slagelse	Died	S	S	S	14	20	11	32	9	7	8	12	32	
SSI-32[UHM]	21-06-13	BAL	Nørre Alslev	Alive	S	S	S	18	23	15	33	11	18	13	9	8	
SSI-58[HCL]	27-09-13	BAL	Sorø	Alive	S	S	S	23	23	15	36	11	20	13	9	5	
ROS-13-6	July 2013	Air	Roskilde	NA	S	S	S	23	22	15	48	11	7	13	9	5	
ROS-13-5	July 2013	Air	Roskilde	NA	S	S	S	21	22	9	12	10	27	10	9	10	
SSI-44[BL]	22-05-13	BAL	Boeslunde	Alive	S	S	S	18	12	8	29	10	20	9	9	8	
SSI-26[UHM]	02-10-13	BAL	Køge	Alive	S	S	S	18	12	11	16	10	13	8	9	5	
ROS-13-7	July 2013	Air	Roskilde	NA	S	S	S	18	12	16	25	10	23	8	9	7	
ROS-13-4	July 2013	Air	Roskilde	NA	S	S	S	18	12	21	28	10	20	8	9	10	
ROS-13-3	July 2013	Air	Roskilde	NA	S	S	S	13	10	9	10	11	9	8	9	19	
SSI-33[Haem.]	22-06-13	BAL	Slagelse	Alive	S	S	S	25	20	8	10	10	21	9	10	5	
ROS-13-2	July 2013	Air	Roskilde	NA	S	S	S	15	20	9	10	10	6	8	10	10	

**Superscript letters indicate underlying diseases of patients, PBL: Plasmablastic lymphoma, BL: Burkitt's lymphoma, UHM: unspecified haematological malignancy, HCL: Hairy cell leukaemia. Haem: haemophagocytosis.*
TS: tracheal/sputum. BAL: bronchoalveolar lavage. Air: air samples from ventilation filters. STRAf: Short tandem repeat A. fumigatus microsatellite genotyping assay. Markers are 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B and 4C. ITZ: Itraconazole, POS: Posaconazole, VRZ: Voriconazole. Dendrogram was created by UPGMA clustering of categorical (STRAf) values. Fractions indicate number of identical markers out of 9 total.

This case illustrated the complexity of *A. fumigatus* outbreak investigation, which due to the ubiquitous nature of *A. fumigatus*, uncovering a source of outbreaks may be like finding a needle in a haystack. The challenge in assessing STRAf genotype data may be associated with further ambiguity, prompted by the finding of identical genotypes from different sources. Indeed, we found a relatively high occurrence of shared genotypes both among Danish clinical and environmental isolates but also across different countries (Table 12). One main point was the scrutiny and care, with which STRAf genotyping data should be interpreted. Identical genotypes could in some settings indicate outbreaks, which is the strength of such high-resolution assay [214] but in our case, the diversity of genotypes in the filters and patients prohibited identification of a common source. On the other hand, identical genotypes were found among patients, which were hospitalised simultaneously at the same wards, and the patient samples handled in the same clinical microbiology laboratory. As only one patient in each incident had signs and symptoms of invasive aspergillosis only one patient was regarded truly infected, while the false positive patients were examples of pseudo-outbreaks [225].

Table 12. Collection of *A. fumigatus* isolates with identical genotypes from unrelated sources.

Group no.	Patient	Date	Site	STR*Af* markers									Azole resistance	Location	100% matching STR*Af* genotypes	
				2A	2B	2C	3A	3B	3C	4A	4B	4C	*CYP51A* profiles	City/country	DK isolate	Origin of foreign isolate
1	SSI-58	Sep-13	BAL	23	23	15	36	11	20	13	9	5	ND Suscept.	Sorø**	KLM-R6	
	KLM-R6	Aug-07	Resp.	23	23	15	36	11	20	13	9	5	WT	Faroe Islands[K]	SSI-58	
2	SSI-53	Jun-14	Exp.	20	21	12	83	10	7	8	9	10	TR_{34}/L98H	København*	SSI-27	
	SSI-53	Aug-14	Exp.	20	21	12	83	10	7	8	9	10	TR_{34}/L98H	København*	SSI-27	
	SSI-27	Jun-14	BAL	20	21	12	83	10	7	8	9	10	TR_{34}/L98H	Odder*	SSI-53	
	SSI-36	Jun-14	Exp.	20	21	12	**84**	10	7	8	9	10	TR_{34}/L98H	Middelfart*	(SSI-27, SSI-53)	
3	KLM-R2	Oct-07	Resp.	14	20	8	40	9	11	8	10	20	TR_{34}/L98H/S297T /F495I	Hillerød*	KLM-R5	Dutch resistant clinical isolate[193]
	KLM-R5	Oct-07	Resp.	14	20	8	40	9	11	8	10	20	TR_{34}/L98H/S297T /F495I	Værløse*	KLM-R2	Dutch resistant clinical isolate[193]
4	AST-4	Mar-14	BAL	18	12	11	16	10	13	8	9	5	WT	Ry***	SSI-26	Bern[214], Apeldoorn
	SSI-26	Oct-13	BAL	18	12	11	16	10	13	8	9	5	ND Suscept.	Køge**	AST-4	Bern[214], Apeldoorn
5	SSI-33	Jun-13	BAL	25	20	8	10	10	21	9	10	5	ND Suscept.	Slagelse**	SSI-18	Oslo[197], Apeldoorn
	SSI-18	Nov-09	Resp.	25	20	8	10	10	21	9	10	5	WT-RES	Halsnæs	SSI-33	Oslo[197], Apeldoorn
6	AST-4	Feb-14	BAL	18	12	8	27	10	20	9	9	5	WT	Ry***	RH-14-4	Merelbeke[226], Nijmegen, Oslo[197]
	RH-14-4	Sep-14	Air	18	12	8	27	10	20	9	9	5	ND Suscept.	København	AST-4	Merelbeke[226], Nijmegen, Oslo[197]
7	KRO-H2	Sep-13	Soil	13	20	10	10	10	10	8	9	20	WT	Ringsted	SSI-8, RH-14-20	Madrid[227], Apeldoorn
	SSI-8	1992	Exp.	13	20	10	10	10	10	8	9	20	ND Suscept.	København	KRO-H2, RH-14-20	Madrid[227], Apeldoorn
	RH-14-20	Sep-14	Air	13	20	10	10	10	10	8	9	20	ND Suscept.	København	KRO-H2, SSI-8	Madrid[227], Apeldoorn
8	AST-3	Jun-13	BAL	20	20	28	32	9	6	8	10	20	TR_{34}/L98H	Kgs. Lyngby		Dutch resistant clinical isolate[193]

Group no.	Patient	Date	Site	STR*Af* markers									Azole resistance	Location	100% matching STR*Af* genotypes	
				2A	2B	2C	3A	3B	3C	4A	4B	4C	*CYP51A* profiles	City/country	DK isolate	Origin of foreign isolate
	ROS-13-3	2013	Air	13	10	9	10	11	9	8	9	19	ND Suscept.	Roskilde**		Merelbeke[226], US [172]
	SSI-44	May-13	BAL	18	12	8	29	10	20	9	9	8	ND Suscept.	Boeslunde**		Apeldoorn
	AST-1	May-12	BAL	18	19	8	26	10	21	9	9	5	WT	Køge		Oslo[197]
	SSI-38	Jan-07	Resp.	18	21	15	60	11	24	17.3	9	5	WT-RES	Hellerup		Würzburg[214]
	SSI-32	Jun-13	BAL	18	23	15	33	11	18	13	9	8	ND Suscept.	Nørre Aslev**		Würzburg[214]
	RH-14-7	Sep-14	Air	20	12	9	10	8	10	8	9	10	ND Suscept.	København		Oslo[197], Apeldoorn
	SSI-20	Nov-10	Exp.	20	19	8	31	14	20	9	9	5	WT-RES	København		Oslo[197]
	AST-2	Jan-13	BAL	14	20	11	34	9	7	8	10	12	WT	Vejle		Oslo[197], Nijmegen, Apeldoorn
	RH-14-12	Aug-14	Air	18	12	13	14	10	12	8	9	5	ND Suscept.	København		Oslo[197], Bern[214], Apeldoorn
	SSI-13	1997	Eye	18	12	21	13	9	17	8	9	10	ND Suscept.	Stenløse		US [172]
	ROS-13-4	2013	Air	18	12	21	28	10	20	8	9	10	ND Suscept.	Roskilde**		Apeldoorn
	RH-14-24	Aug-14	Air	21	22	18	26	10	15	9	13	8	ND Suscept.	København		Apeldoorn
	SSI-47	Mar-13	Exp.	18	23	26	36	11	28	22	10	8	WT-RES	Risskov		Apeldoorn

**Presumed pseudo-outbreak. All samples obtained and cultured within 3 days at the same hospital [225].*
***Patients or air samples from the Roskilde outbreak*
****Sample obtained while patient were hospitalized at Rigshospitalet.*
Group 1 through 7 contain two or more isolates with identical genotypes.
KKLM-R6 is a cystic fibrosis patient and regularly in Denmark where respiratory samples are obtained. Whether this isolate is acquired from Denmark is unclear

The fact that more than 15% of unique genotypes among Danish *A. fumigatus* isolates were identical to genotypes from other countries despite the high discriminatory potential of STRAf typing was surprising. Such frequency may support a theory on clonal expansion and especially genotypes from Norway (3.3%) and The Netherlands (1.7%) were relatively frequent shared with Danish genotypes (Figure 21). Importantly, two genotypes from isolates carrying the TR_{34}/L98H resistance mechanisms were shared with Dutch TR_{34}/L98H isolates and it would be especially interesting to analyse such isolates in more depth and determine the degree of relatedness. Whether the resistance mechanisms are constantly evolving in the environment or whether resistant clones are ubiquitously spread may still be questioned. Genetic insight in a Dutch collection of *A. fumigatus* isolates suggested clonal expansion of the $_{TR34}$/L98H clone rather than random sexual reproduction leading to the same resistant phenotype [228]. Although, this study only included isolates from one country, samples were gathered from 300 km apart, which could indicate an airborne spread [228]. Moreover, identical STRAf genotypes with the $_{TR34}$/L98H resistance mechanism distributed all over India were found [189] as well as identical genotypes and resistance mechanisms among isolates from Tanzania and the Netherlands [190]. One study further highlighted the possibility that an airborne route could be responsible for the spread to other nearby countries and continents based on whole genome sequencing of STRAf related TR_{34}/L98H isolates [229]. This study demonstrated very close relationship between the TR_{34}/L98H isolates within India and suggested that such (dominant and stable) clone could have arisen from a recent mating of a stable Indian wild-type strain with a European TR_{34}/L98H isolate [229]. Finally, the authors suggested that despite a higher diversity among European TR_{34}/L98H isolates, one common ancestor could indeed have been the case, potentially arising from The Netherlands [229]. The diversity of *A. fumigatus* and the spread of this organism is yet to be fully understood. Indeed, the reproductive mode and the production of millions of asexual spores underline the potential of clonal expansion of stable clones. Still, the unique feature of this organism to undergo sexual mating does challenge the overall understanding of the spread of *A. fumigatus*. Whole genome sequencing may indeed play a significant role in exploring this complex situation and help elucidate the true origin of environmentally derived resistance mechanisms.

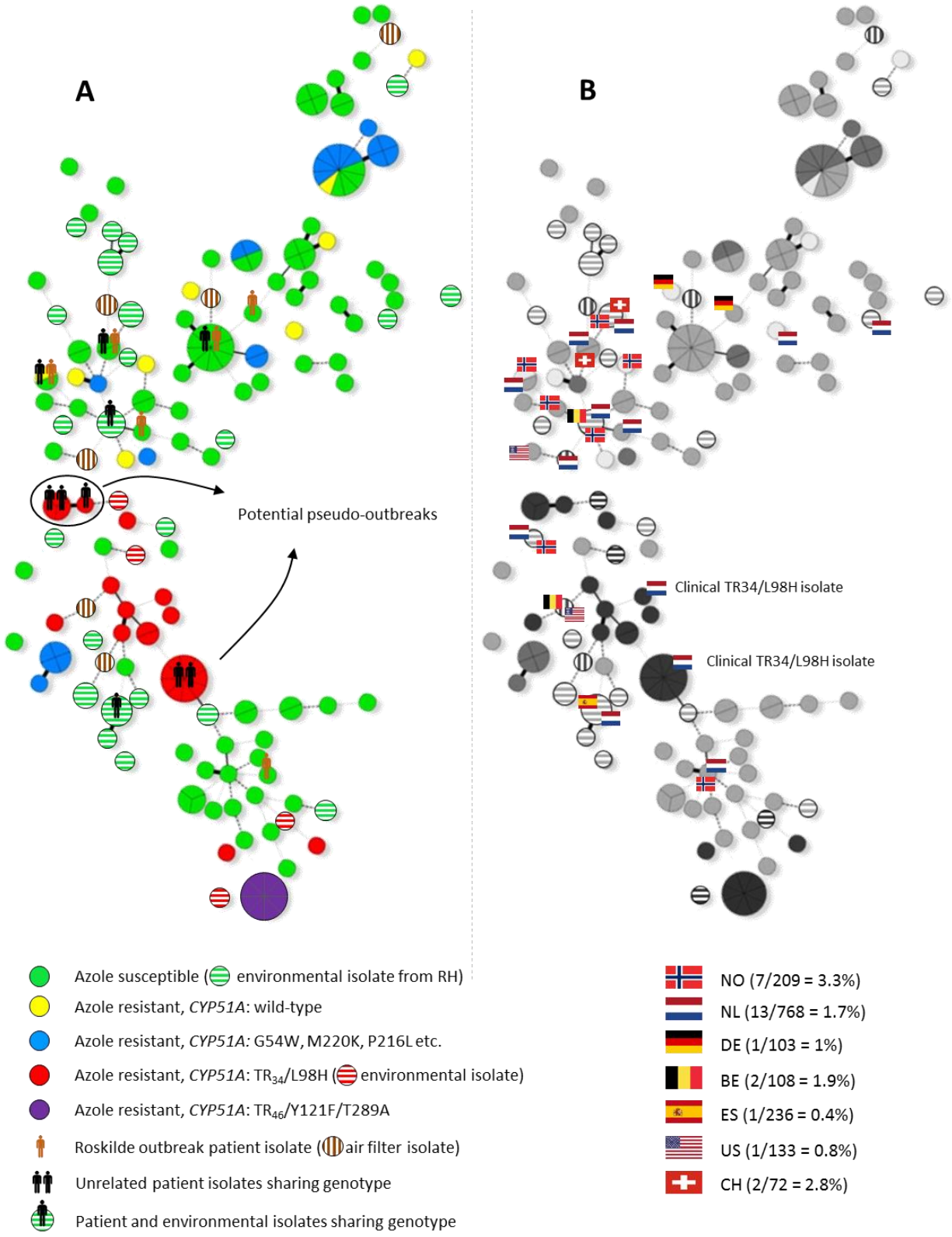


Figure 21. Minimum spanning tree of STRAf genotyped Danish *A. fumigatus* isolates. Each circle represents a unique genotype. Heavy connecting lines indicate similarity in 8/9 microsatellites. Colours, symbols and flags are described in the figure. A) Danish overview illustrating genotypes from azole susceptible and resistant isolates as well as clinical and environmental isolates. Furthermore, the Roskilde outbreak and the seven incidents of shared STRAf genotypes between unrelated isolates have been indicated. RH, University Hospital of Copenhagen, Rigshospitalet. B) Black and white representation of the Danish genotypes with national flags of where *A. fumigatus* has been uncovered sharing identical STRAf genotypes on all 9 markers. The numbers and % in parenthesis is the number of genotypes/total from each country (in our data), which were identical to Danish STRAf genotypes.

PART IV: RECAPITULATION AND PERSPECTIVES

4.1 Recapitulating antifungal drug resistance in Denmark

Drug resistance has emerged in Denmark and is a persisting concern for patients suffering from severe *Candida* and *Aspergillus* infections. Uncovering the true prevalence of antifungal resistance in Denmark is complex but this thesis may contribute to a broader overview of the current situation for the most frequent invasive fungal infections. Indeed, the management of fungal infections face the challenges of resistance and as we demonstrated for *A. fumigatus* also from an external source. For *Candida* infections, the main concern remains a changing epidemiology towards species intrinsically less susceptible to fluconazole. Still, rapidly acquired resistance to echinocandins gives rise to another concern. Among Danish clinical *Candida* isolates, a larger capacity of *C. glabrata* to acquire echinocandin resistance was indicated, posing a potential multi-drug resistant threat due to the inherent reduced fluconazole susceptibility of this species [152]. Indeed *C. glabrata* is suggested to be highly prone to resistance development due to a discernible degree of genomic instability triggered by antifungal stress induction [89]. Multidrug resistance is however rare, probably due to the complexity and multitude of genetic events cellular changes required for such trait [119]. Still, *C. albicans* illustrated an enormous capacity to evolve with antifungal drugs and the proposed novel azole and polyene resistance mechanisms deserve further investigation. Insight in the genetic landscape of antifungal resistance contributes to the understanding of resistance selection and may in turn help preventing resistance development as well as emphasise the demand for novel fungicidal drugs [89]. Antifungal resistance in *Candida* may be more frequent than suggested by surveillance studies and the oral cavity may serve as an unrecognised reservoir for resistance selection, which was also shown in the abdomen in relation to echinocandin resistance [19]. Species distributions affected by antifungal exposure was indicated in this thesis and has been shown previously both for fluconazole and caspofungin treated patients [132, 133, 136]. The clinical implications of such findings imply that any patient exposed to antifungal drugs may have altered *Candida* colonisation, which in turn imposes a risk of later infection caused by a species with intrinsic or acquired resistance. This could be further illuminated by two clinical surveys; one follow up study of survived patients, obtaining oral swabs at later time-points to clarify whether the resistant strain had persisted or only transiently present in response to antifungal exposure. Another study could investigate the oral flora in patients before initiation of empirical treatment and subsequently tracing those patients with documented candidiasis to see whether the preliminary exposure had an effect on the invasive pathogen. Such study would give some clarity to the missing link in our study and is similar to a previous study, except that the number of detected invasive infections were very low [136]. The overall conclusion remains the same; antifungal exposure is a strong driver of resistance in *Candida*emia patients, and may be higher in certain anatomical niches such as the oral microbiota. This underlines the necessity of careful antifungal stewardship as well as susceptibility testing of *Candida* isolates both from blood but ideally also from other sites of the body.

The overall azole resistance rates among clinical *A. fumigatus* isolates in Denmark have increased to about 6% (4% on patient level), and mostly represented by the $_{TR34}$/L98H resistance mechanism as in the Netherlands [176]. Azole resistant *A. fumigatus* derived from environmental fungicide use is a tangible concern and although the incidence is low, we illustrated that this phenomenon is still associated with serious clinical implications. This situation may be somewhat similar to the observed methicillin resistant Staphylococcus aureus (MRSA), emerged in conventional pig farms and now serving as a source of infection [230, 231]. Also here, antibiotic resistance has evolved in the environmental niche (in the pigs) and pose a constant threat to farmers as well as associated families, which are in close contact with MRSA carrying pigs. The severity of this issue is substantiated by the fact that humans can be permanent carriers of MRSA strains, posing a discernible threat if a patient carrying MRSA becomes vulnerable to infection [230]. Although rarer and probably more complex, azole resistant *A. fumigatus* in the environment may pose a comparable serious health concern, especially for those patients at risk of acquiring IA and increased awareness is warranted. Moreover, the supposed outbreak related to renovation of ceilings in nearby hallways underlines the importance of maintaining spore levels in the air of hospital wards (especially haematological and intensive care units) as low as possible [173]. It is possible that the occurrence of *Aspergillus* associated outbreaks is underreported since many IA cases are never diagnosed or remain unresolved. Moreover, extra care should be taken, before reporting on potential airborne outbreaks, in order to avoid undesirable media attention, and improved tools are still demanded for resolving outbreaks of this ubiquitous microbe.

The worldwide spread of azole resistance in the environment still needs further studies but there lies a great challenge in unravelling the spread of potential resistant ancestors due to the clonal as well as sexual reproductive mode and the generation of often millions of progeny within one *A. fumigatus* colony. Our analysis of accumulated Danish genotypes illustrated the enormous diversity of *A. fumigatus* as well as the somewhat ambiguity when interpreting the results. The high degree of identical genotypes in Danish and foreign isolates were unexpected and could be a result of clonal expansion. Despite the high discriminatory power of the microsatellite based STRAf genotyping assay, our results suggest that care, with which interpretation of such data, should be assessed. Nevertheless, early diagnosis, systematic referral of mould isolates as well as susceptibility testing is in high demand to improve the management of invasive aspergillosis. This would further enhance the epidemiological understanding and the constant monitoring of the emerging azole resistance in Denmark.

4.2 Relevant topics left behind

In the attempt in covering the broad perspectives of antifungal drug resistance in Denmark, the compromise has been to stay rather brief on certain topics, which may be more significant than accounted for here. First, *Candida* biofilm formation especially on foreign objects such as catheters not only serves as a major source for disseminated *Candida* infection but may also serve as first line resistance against antifungals [232]. Moreover, biofilm may further enable resistance development due to associated sub-therapeutic drug concentrations within the biofilm [232]. The genetic interplay and cellular components involved in biological challenging feature is extensive and not fully elucidated [233]. The cellular landscape and genetic mechanisms involved in resistance may be even more complex than addressed here and indeed the novelty of potential resistance mechanisms require independent validation. Moreover, explaining why virulence was less attenuated in the MDR *C. albicans* isolates deserve further

elucidation and by doing so, cellular and genetic mechanisms may be identified, which could serve as potential targets for reducing virulence. One antifungal drug class not discussed in this thesis is the pyrimidine analogue 5-flucytosine (a prodrug), which is a drug inhibiting both protein and DNA synthesis mediated by intracellular modifications. This drug is rarely used as monotherapy due to a high level of acquired resistance when given alone but have served applicable in rare *Candida* infections e.g. at challenging anatomical sites such as the central nervous system [234, 235]. Still, resistance to 5-flucytosine is primarily driven by mutations leading to specific amino acid substitutions in proteins involved in uptake and modifications of the prodrug [235].

4.3 Three areas requiring further research

There are several projects, which are currently ongoing and require further research (Figure 22).

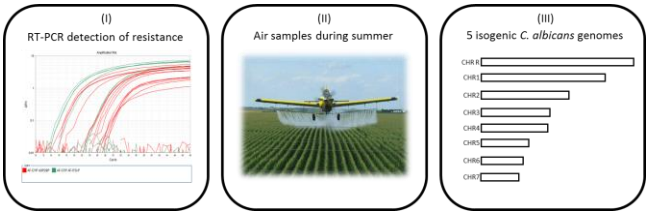


Figure 22. Three projects to be completed in near future. Left panel, RT-PCR to detect *A. fumigatus* in respiratory samples as well as the two environmental resistance markers. Mid panel, spore collection from air during summer months from agricultural fields. Right panel, whole genome sequencing of the 5 sequential and supposedly isogenic *C. albicans* strains from Paper III, to uncover the full genetic landscape responsible for the observed resistance.

(I) Real-time PCR based analysis for the detection of *Aspergillus* DNA among respiratory samples is currently in the pipeline and may serve as a valuable contribution to improved diagnostics of IA [236]. This includes detection of the two environmentally derived resistance mechanisms TR_{34}/L98H and TR_{46}/Y121F/T289A, which may further help resolve the choice of treatment, when aspergillosis is suspected. Similar approaches have been proposed for echinocandin resistance in *C. albicans* targeting *FKS1* [237, 238]. The panel of PCR assays may be expanded to *C. glabrata* too but as this thesis also demonstrated, *FKS1* variants are diverse and still emerging, thus, assays for all *FKS1* variants in *Candida* would be infeasible.

(II) Azole resistant *A. fumigatus* remained absent in our environmental surveys but sampling during the summer period may help elucidate the true prevalence of TR_{34}/L98H and TR_{46}/Y121F/T289A strains in the environment. One ongoing environmental study is been carried out by a collaborating agricultural university and they have collected spores during the summer months June-August for several years and from several conventional agricultural fields. DNA will be extracted from these samples and analysed in the above mentioned RT-PCR for the detection of *A. fumigatus* as well as azole resistance markers.

(III) Further in depth understanding of the MDR *C. albicans* isolates from Paper III has been sought by whole genome sequencing. Thus the entire genetic landscape may be elucidated and the underlying chromosome transformations, involved in loss-of-heterozygosity of both *ERG11* and *TAC1*, uncovered [102]. Moreover, single nucleotide polymorphism (SNP) analysis would help reveal the rate of which spontaneous mutations occurred during the heavy antifungal pressure that the patient was exposed to.

4.4 Three suggested future projects

Besides the ongoing projects, other ideas have emerged throughout this thesis and may be pursued in the future (Figure 23).

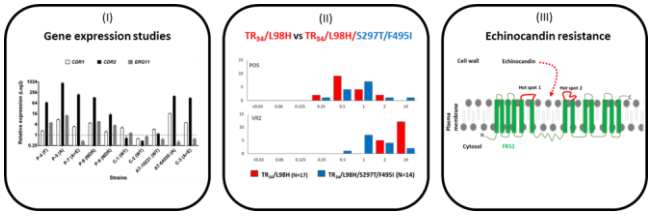


Figure 23. Three suggested projects for future studies. Left panel, gene expression studies in relation to drug resistance, particularly azole resistance in *Candida* species. Mid panel, assessment of potential differences between the two variants of TR_{34}/L98H, which could have clinical relevance. Right panel, echinocandin resistance at certain anatomical niches could be investigated further.

(I) Gene expression study. Elevated expression levels of genes (applied in Paper III) have thus far been carried out by collaborators, despite that this cellular feature plays a significant role in potentiating azole resistance especially for *C. glabrata*. One outcome of the national surveillance as well as the clinical study in Paper IV was the high number of azole resistant *Candida* and the underlying mechanisms remain to be fully resolved in these isolates. While the methodology may be straight-forward we still do not have this analysis implemented and would be desirable [122].

(II) Further assessment of the TR_{34}/L98H/S297T/F495I mechanism. Indications on slightly different susceptibility profiles of the TR_{34}/L98H vs. the other variant TR_{34}/L98H/S297T/F495I was observed (supplementary reading, S.7 Azole MIC distribution of frequent *CYP51A* variants). Intuitively, additional amino acid substitutions in *CYP51A* could have implications on azole susceptibility but not necessarily increasing resistance. Instead, the TR_{34}/L98H/S297T/F495I variant displayed a modal MIC one step higher for posaconazole but two steps lower for voriconazole compared to TR_{34}/L98H. Indeed, batch to batch variations, biological inaccuracy, low number of isolates etc. may all contribute to an inaccurate interpretation of this observation but it may be relevant to pursue. This could have significant clinical implication in the management of such infections because a lower in vitro MIC for voriconazole could potentially correlate to successful management by dose escalation of voriconazole rather than alternating treatment to weaker alternatives.

(III) Further assessment of the in vivo role of echinocandins. Investigating echinocandin penetration in different relevant anatomical sites applying pharmacodynamics and pharmacokinetics could be pursued. This would involve improved measurements of echinocandin concentrations in those sites compared to serum levels. Moreover, such study should also address the potentially increased risk of acquired resistance as a consequence of sub-therapeutic drug levels in those anatomical sties. The protein bound nature of echinocandins may indeed be the primary cause of sub-therapeutic levels in cer-

tain sites and studying this feature could help identify the required levels for efficient treatment in those anatomical sites.

4.5 Ideas for future

If money, time, expertise and hands were not an issue, the number of ideas for future studies are many and I have sought to outline the concepts of those, I find most relevant (Figure 24).

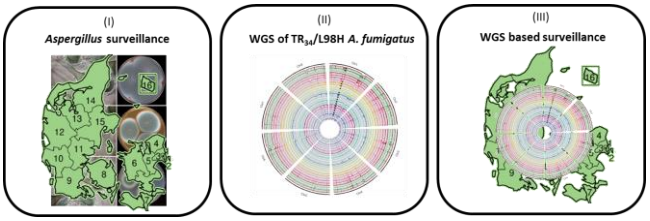


Figure 24. Ideas for the future. Left panel, national surveillance of invasive *Aspergillus* infections. Mid panel, routine whole genome sequencing of all collected *A. fumigatus* isolates harbouring environmental azole resistance mechanisms. Right panel, WGS based surveillance of all invasive fungal infections.

(I) As indicated for the *Aspergillus* section, a systematic referral of mould isolates would increase the detection of *Aspergillus* infections as well as the precision of estimated resistance rates. Hopefully, such national surveillance could be implemented. This should ideally be combined with an improved evaluation of respiratory and one day serum samples from high-risk patients to be analysed by *Aspergillus* specific PCR in addition to current biomarker assays. This would contribute to improved diagnostics and thus patient care, a refined understanding of the actual burden not only of invasive aspergillosis but also of environmental azole resistance rates.

(II) Whole genome sequencing of isolates carrying TR_{34}/L98H and TR_{46}/Y121F/T289A resistance mechanisms could help elucidate the origin and route of these clones and answer one important question. Are these clones dispersed by clonal expansion or are they independently developed in different niches all around the globe?

(III) In the bacterial world, smaller genomes and primarily clonal expansion make WGS invaluable in studying these organisms both with regards to outbreak investigation, resistance screening, virulence determination and epidemiological purposes. Likewise, such advanced methods could one day be applied in combination with routine surveillance of yeast and mould infections and a national WGS based surveillance of all invasive fungal infections could be a highly valuable tool. Still, the fungal genomes are typically 10 times larger than bacteria and combined with additional factors such as sexual mating and poly-ploidy of fungal species make such approach currently less attractive. When more fungal genomes are mapped, reliable databases curated and most genes concerning resistance and virulence have been identified, this platform may one day become economically feasible.

On an even larger scale other ideas for future projects and collaborations are listed below.

- Genetic platform for mutational analyses. The *ERG2* mutation was sought constructed in *C. albicans* (and later S. cerevisiae as a model) at another collaborating university both by me but also a master's student, although both unsuccessfully. I therefore propose the establishment of a high throughput genetic platform for the in vitro evidence based assessment of potentially novel resistance associated mutations. Such platform is currently present in other labs and should be within reach. The platform should primarily be within *Candida* but an equivalent platform for *A. fumigatus* would also be desired.
- Surveillance of invasive fungal infections is not permanently implemented in many fellow Nordic countries and thus initiatives such as a Scandinavian fungaemia surveillance network could be warranted. This could help elucidate the low incidence of *Candida*emia outside Denmark and also further address whether antifungal resistance may be overlooked in other countries.
- Microbiome studies are increasingly employed and the host mycobiome in relation to gastrointestinal diseases have been investigated [239]. Moreover, microbiome studies based on bacteria (using the 16S ribosomal DNA) and fungi (using the ITS region) are available but it would be intriguing to pursue a combined bacterial and eukaryotic microbiome assay (bacteria, parasites and fungi). This could have extensive clinical as well as scientific potential since it is now acknowledged, that the microbiome play vital roles both in relation to infections but also in a wide range of non-microbial diseases and lifestyle syndromes [240].

4.5 The final note

Please remember three points from this thesis.

(I) Antifungal exposure is associated with the risk of selecting resistance, primarily against echinocandins in *Candida*, but the changing epidemiology may pose the most apparent concern.

(II) Antifungal resistance may be underestimated among drug exposed patients and thus, precautions should be taken when managing fungal infections, especially in patients previously exposed to antifungals.

(III) Long-term azole exposure in patients with *A. fumigatus* infections may select resistance in vivo but the most important clinical concern is the increased risk of acquiring azole resistant *A. fumigatus* derived from environmental fungicide.

4.6 Acknowledgements

This work has not been possible without the extraordinary supervision by Professor Maiken Cavling Arendrup, essential guidance by Dr. Med. Helle Krogh Johansen, laboratory aid by Birgit Brandt and the entire mycology laboratory at Statens Serum Institut (SSI), as well as PCR help by Gitte Jensen, Lis Wassman, Mette Kjærgaard and numerous inspiring and helpful colleagues at Microbiology and Infection Control, SSI. Moreover, invaluable collaborations around the globe covering Professor David Perlin's laboratory, with additional thanks to PhD. Cristina Ortega at the Public Health and Research Instititute, now Rutgers University, USA, Dr. Med. Jacques Meis and PhD. Ferry Hagen and the laboratory staff at the Canisius-Wilhelmina Ziekenhuis, the Netherlands and Associate Professor Dominique Sanglard and PhD. Luis Vale Silva from the Centre Hospitalier Universitaire Vaudois, Switzerland. Danish collaboration includes Professor Uffe H. Mortensen, PhD. Dorte Pedersen and the staff at the Department of Systems Biology at the Technical University of Denmark as well as Senior Researcher Lise Nistrup Jørgensen and PhD fellow Thies Marten Wieczorek at Flakkebjerg, Aarhus University. Christina Brandt Andersen is thanked for her brilliant assistance for the PhD sub-

mission to the DMJ and on a personal level, Mette Krøger is forever thanked for her endless love and support, Karen Astvad and Klaus Mortensen for their mutual struggles as fellow PhD. Colleagues, and finally so many thanks to my family and friends for bystanding the entire process.

ABSTRACT

Antifungal drug resistance is a multifaceted clinical challenge, and when present, a primary cause of treatment failure in patients with severe fungal infections. Changing epidemiology, increasing resistance rates and a narrow antifungal armamentarium may further underline the required attention on resistance particularly within the most prevalent invasive fungal infections caused by *Candida* yeasts and *Aspergillus* moulds. In Denmark, the resistance epidemiology remains to be fully elucidated. This thesis sought to address this demand as well as provide insight into the landscape of underlying molecular resistance mechanisms.

Paper I and II both contributed to the understanding of *FKS* (β-glucan synthase) mediated echinocandin resistance in *Candida* species. Paper I covered a unique stepwise acquisition of a homozygous mutation in *FKS1* of *Candida tropicalis* leading to an amino acid change corresponding to a well-known S645P in *Candida albicans*. Paper II presented a failure case due to *Candida krusei* displaying high-level echinocandin resistance likely attributable to an acquired D662Y amino acid substitution in *FKS1*. Intrinsic differences in *FKS1* among *Candida* species may explain why the level of resistance both depends on the mutation as well as the species and cannot be easily translated to the level of clinical resistance. Intrinsic fluconazole resistance in *C. krusei* further substantiated the clinical implications of acquired echinocandin resistance.

Paper III presented a rare multidrug resistance case in a series of isogenic *C. albicans* isolates, almost covering the entire spectrum of known resistance mechanisms in *Candida* and involved the proposal of novel resistance mutations. An A61E change in *ERG11* was potentially involved in reduced susceptibility to long-structured azoles. Increased expression levels of azole efflux pumps were probably accredited to novel gain-of-function variants in the transcription factor *TAC1* (R688Q and R673L). Echinocandin resistance was induced by the well-known S645P variant of *FKS1* and polyene resistance was likely inflicted by a frameshift mutation in *ERG2* leading to loss of function of the encoded protein and subsequent ergosterol depletion.

The number of acquired resistance cases is increasing in our settings and Paper IV sought to illuminate whether antifungal resistance is overlooked in the current fungaemia programme. This involved the acquisition of post-treatment oral isolates from 193 Candidaemia patients among which 114 received azoles (primarily fluconazole) and 85 received an echinocandin (and some both). Azole exposed patients carried a significantly higher proportion of species less susceptible to fluconazole (primarily *Candida glabrata*) among colonising *Candida* compared to baseline blood isolates (P<0.001). A similar trend was seen for echinocandin treated patients although not statistically significant. Interestingly, there was a high frequency of acquired resistance, 29.4% to fluconazole and 21.6% to echinocandins, among colonising *C. glabrata* isolates post treatment. These figures were both significantly higher compared to baseline blood isolates as well as oral isolates from patients with no or minimal exposure to either drug class. In contrast, acquired resistance among *C. albicans* oral isolates was rare (<5%). Thus, the oral cavity may be an unrecognized reservoir of resistant *Candida* species, especially *C. glabrata* following azole or echinocandin treatment. This underlines the care of which therapeutic stewardship must be taken both for antifungal naïve patients, to avoid resistance development, as well as for patients previously exposed to antifungals.

Paper V presented four fatal cases of invasive aspergillosis involving azole resistant *Aspergillus fumigatus* harbouring resistance mechanisms (TR_{34}/L98H and TR_{46}/Y121F/T289A), which are thought to derive from environmental fungicide use. The clinical concern is evident because the route of infection is through inhalation of potentially azole resistant spores. Still, recent environmental surveys were unable to detect azole resistant *A. fumigatus* in numerous soil samples but seasonal variations could be one explanation for this paradox.

Paper VI was a retrospective laboratory based study and aimed to elucidate the prevalence of azole resistance in *A. fumigatus* isolates from 2010-2014 in Denmark. This study also sought to uncover the underlying resistance mechanisms, primarily attributable to *CYP51A* mutations, and finally to assess the accumulated genotyping data. Among 1162 *A. fumigatus* isolates, 94.5% were screened for azole resistance and a significant increasing trend was observed for the number of azole resistant isolates to approximately 6% in 2014 (P<0.001) and 4% in corresponding patients (P<0.05). The underlying resistance mutations were diverse but still dominated by the TR_{34}/L98H resistance mechanism responsible for >50% of all our azole resistant isolates. The genotyping data of resistant and a selection of susceptible *A. fumigatus* showed high identity to foreign isolates (>15%). This could argue for the hypothesis on clonal expansion, which has previously been suggested for TR_{34}/L98H clones in the Netherlands and India, but could also indicate an insufficient discriminatory power of such analysis. Still, a proposed *A. fumigatus* outbreak in a haematology ward was unresolved since no genetically identical isolates were recovered from patients and air samples, illustrating the ubiquitous nature of this organism.

Overall, the main concerns are a changing *Candida* epidemiology towards species less susceptible to fluconazole combined with the rapid acquisition of echinocandin resistance, especially among *C. glabrata* isolates. For *A. fumigatus*, the concern is the emergence of azole resistant strains in the environment, displaying cross-resistance to clinical azoles, and thus posing unforeseen clinical challenges in the management of invasive aspergillosis. Collectively, these findings call for an increased awareness especially at clinical microbiology laboratories, which ideally would lead to susceptibility testing of all clinically relevant isolates by reference or validated methods. Moreover, novel diagnostic approaches for non-culturable pathogens are warranted and especially DNA based detection by PCR may serve as a solid complimentary tool for improved diagnostics of invasive fungal infections.

REFERENCES

1. Darwin C. 1859. On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life, 1st ed. John Murray, Albemarle Street, London.
2. Snelders E, van der Lee HAL, Kuijpers J, Rijs AJMM, Varga J, Samson RA, Mellado E, Donders ART, Melchers WJG, Verweij PE. 2008. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. PLoS Med 5:e219.

3. Pfaller MA, Diekema DJ. 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 20:133–63.
4. Denning DW. 1996. Aspergillosis: diagnosis and treatment. Int J Antimicrob Agents 6:161–8.
5. Pfaller MA. 2012. Antifungal drug resistance: mechanisms, epidemiology, and consequences for treatment. Am J Med 125:S3–13.
6. Kathuria S, Singh PK, Sharma C, Prakash A, Masih A, Kumar A, Meis JF, Chowdhary A. 2015. Multidrug resistant *Candida auris* misidentified as C. haemulonii : Characterization by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), DNA sequencing and . J Clin Microbiol 53:JCM.00367–15.
7. Sanglard D, Ischer F, Parkinson T, Falconer D, Bille J. 2003. *Candida albicans* mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. Antimicrob Agents Chemother 47:2404–12.
8. Sanglard D, Ischer F, Koymans L, Bille J. 1998. Amino Acid Substitutions in the Cytochrome P-450 Lanosterol 14alpha-Demethylase (*CYP51A*1) from Azole-Resistant *Candida albicans* Clinical Isolates Contribute to Resistance to Azole Antifungal Agents. Antimicrob Agents Chemother 42:241–253.
9. Pappas PG, Kauffman CA, Andes D, Benjamin DK, Calandra TF, Edwards JE, Filler SG, Fisher JF, Kullberg B-J, Ostrosky-Zeichner L, Reboli AC, Rex JH, Walsh TJ, Sobel JD. 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis 48:503–35.
10. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Segal BH, Steinbach WJ, Stevens DA, van Burik J-A, Wingard JR, Patterson TF. 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 46:327–60.
11. Perlin DS. 2014. Current perspectives on echinocandin class drugs. Future Microbiol 6:441–457.
12. Kartsonis NA, Nielsen J, Douglas CM. 2003. Caspofungin: The first in a new class of antifungal agents. Drug Resist Updat 6:197–218.
13. Walker LA, Gow NAR, Munro CA. 2010. Fungal echinocandin resistance. Fungal Genet Biol 47:117–26.
14. Gray KC, Palacios DS, Dailey I, Endo MM, Uno BE, Wilcock BC, Burke MD. 2012. Amphotericin primarily kills yeast by simply binding ergosterol. Proc Natl Acad Sci U S A 109:2234–9.
15. Alonso MAS, Ramos IJ, Lleti MS, Peman J. 2006. Epidemiology of invasive fungal infections due to *Aspergillus* spp . and Zygomycetes. Clin Microbiol Infect 12:2–6.
16. Alastruey-Izquierdo A, Castelli MV, Cuesta I, Monzon A, Cuenca-Estrella M, Rodriguez-Tudela JL. 2009. Activity of posaconazole and other antifungal agents against Mucorales strains identified by sequencing of internal transcribed spacers. Antimicrob Agents Chemother 53:1686–9.
17. Maertens J, Marchetti O, Herbrecht R, Cornely OA, Flückiger U, Frêre P, Gachot B, Heinz WJ, Lass-Flörl C, Ribaud P, Thiebaut A, Cordonnier C. 2011. European guidelines for antifungal management in leukemia and hematopoietic stem cell transplant recipients: summary of the ECIL 3--2009 update. Bone Marrow Transplant 46:709–18.
18. Arendrup MC. 2013. *Candida* and *Candida*emia. Susceptibility and epidemiology. Dan Med J 60:B4698.
19. Shields RK, Nguyen MH, Press EG, Clancy CJ. 2014. Abdominal candidiasis is a hidden reservoir of echinocandin resistance. Antimicrob Agents Chemother 58:7601–5.
20. Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope W, (EUCAST)* S on AST (AFST) of the EE for AST. 2012. EUCAST technical note on the EUCAST definitive document EDef 7.2: Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts EDef 7.2 (EUCAST-AFST). Clin Microbiol Infect 18:E246–247.
21. Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope W, Howard SJ, (EUCAST)* S on AST (AFST) of the EE for AST. 2009. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. Clin Microbiol Infect 15:103.
22. CLSI. 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard-Third Edition, M27–A3 ed. Clinical and Laboratory Standards Institute, Wayne, PA.
23. CLSI. 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard-Third Edition, M27–S4 ed. Clinical and Laboratory Standards Institute, Wayne, PA.
24. Arendrup M, Horn T, Frimodt-Møller N. 2002. In vivo pathogenicity of eight medically relevant *Candida* species in an animal model. Infection 30:286–91.
25. Koga-Ito CY, Komiyama EY, de Paiva Martins CA, Vasconcellos TC, Cardoso Jorge AO, Carvalho YR, do Prado RF, Balducci I. 2011. Experimental systemic virulence of oral *Candida* dubliniensis isolates in comparison with *Candida albicans*, *Candida tropicalis* and *Candida krusei*. Mycoses 54:7–12.
26. Moran GP, Coleman DC, Sullivan DJ. 2011. Comparative Genomics and the Evolution of Pathogenicity in Human Pathogenic Fungi. Eukaryot Cell 10:34–42.
27. Mortensen KL, Johansen HK, Fuursted K, Knudsen JD, Gahrn-Hansen B, Jensen RH, Howard SJ, Arendrup MC. 2011. A prospective survey of *Aspergillus* spp. in respiratory tract samples: prevalence, clinical impact and antifungal susceptibility. Eur J Clin Microbiol Infect Dis 30:1355–63.
28. Berdal JE, Haagensen R, Ranheim T, Bjørnholt J V. 2014. Nosocomial candidemia; risk factors and prognosis revisited; 11 years experience from a Norwegian secondary hospital. PLoS One 9:5–10.
29. Sipsas N V., Lewis RE, Tarrand J, Hachem R, Rolston K V., Raad II, Kontoyiannis DP. 2009. Candidemia in patients with hematologic malignancies in the era of new antifungal agents (2001-2007): Stable incidence but changing epidemiology of a still frequently lethal infection. Cancer 115:4745–4752.
30. Puig-Asensio M, Pemán J, Zaragoza R, Garnacho-Montero J, Martín-Mazuelos E, Cuenca-Estrella M, Almirante B, Prospective Population Study on Candidemia in Spain (CANDIPOP) Project, Hospital Infection Study Group (GEIH), Medical Mycology Study Group (GEMICOMED) of the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC), Spanish Network for Research in Infectious Diseases. 2014. Impact of therapeutic strategies on the prognosis of candidemia in the ICU. Crit Care Med 42:1423–32.
31. Arendrup MC, Dzajic E, Jensen RH, Johansen HK, Kjaeldgaard P, Knudsen JD, Kristensen L, Leitz C, Lemming LE, Niel-

sen L, Olesen B, Rosenvinge FS, Røder BL, Schønheyder HC. 2013. Epidemiological changes with potential implication for antifungal prescription recommendations for fungaemia: data from a nationwide fungaemia surveillance programme. Clin Microbiol Infect 19:E343–53.
32. Arendrup MC, Sulim S, Holm A, Nielsen L, Nielsen SD, Knudsen JD, Drenck NE, Christensen JJ, Johansen HK. 2011. Diagnostic issues, clinical characteristics, and outcomes for patients with fungemia. J Clin Microbiol 49:3300–8.
33. Lau A, Sorrell TC, Chen S, Stanley K, Iredell J, Halliday C. 2008. Multiplex tandem PCR: a novel platform for rapid detection and identification of fungal pathogens from blood culture specimens. J Clin Microbiol 46:3021–7.
34. Quindós G. 2014. Epidemiology of *Candida*emia and invasive candidiasis. A changing face. Rev Iberoam Micol 31:42–48.
35. Lewis JS, Wiederhold NP, Wickes BL, Patterson TF, Jorgensen JH. 2013. Rapid Emergence of Echinocandin Resistance in *Candida glabrata* Resulting in Clinical and Microbiologic Failure. Antimicrob Agents Chemother 57:4559–61.
36. Douglas CM, D'Ippolito JA, Shei GJ, Meinz M, Onishi J, Marrinan JA, Li W, Abruzzo GK, Flattery A, Bartizal K, Mitchell A, Kurtz MB. 1997. Identification of the *FKS1* gene of *Candida albicans* as the essential target of 1,3-beta-D-glucan synthase inhibitors. Antimicrob Agents Chemother 41:2471–9.
37. Johnson ME, Edlind TD. 2012. Topological and Mutational Analysis of Saccharomyces cerevisiae *FKS1*. Eukaryot Cell 11:952–60.
38. Perlin DS, Shor E, Zhao Y. 2015. Update on Antifungal Drug Resistance. Curr Clin Microbiol Reports 2:84–95.
39. Baixench M-T, Aoun N, Desnos-Ollivier M, Garcia-Hermoso D, Bretagne S, Ramires S, Piketty C, Dannaoui E. 2007. Acquired resistance to echinocandins in *Candida albicans*: case report and review. J Antimicrob Chemother 59:1076–83.
40. Hernandez S, Lo L, Najvar LK, Mccarthy DI, Bocanegra R, Graybill JR. 2004. Caspofungin Resistance in *Candida albicans* : Correlating Clinical Outcome with Laboratory Susceptibility Testing of Three Isogenic Isolates Serially Obtained from a Patient with Progressive *Candida* Esophagitis. Antimicrob Agents Chemother 48:1382–1383.
41. Park S, Kelly R, Kahn JN, Robles J, Hsu M, Register E, Li W, Vyas V, Fan H, Abruzzo G, Flattery A, Gill C, Chrebet G, Parent SA, Kurtz M, Teppler H, Douglas CM, Perlin DS. 2005. Specific Substitutions in the Echinocandin Target *FKS1*p Account for Reduced Susceptibility of Rare Laboratory and Clinical *Candida* sp . Isolates. Antimicrob Agents Chemother 49:3264–3273.
42. Laverdière M, Lalonde RG, Baril J-G, Sheppard DC, Park S, Perlin DS. 2006. Progressive loss of echinocandin activity following prolonged use for treatment of *Candida albicans* oesophagitis. J Antimicrob Chemother 57:705–8.
43. Dannaoui E, Desnos-Ollivier M, Garcia-Hermoso D, Grenouillet F, Bretagne S, Dromer F, Lortholary O. 2012. *Candida* spp. with Acquired Echinocandin Resistance, France, 2004-2010. Emerg Infect Dis 18:86–90.
44. Daneman N, Chan AK, Poutanen SM, Rennie R, Sand C, Porter S. 2006. The emergence of caspofungin resistance during treatment of recurrent *Candida glabrata Candida*emia. Clin Microbiol Infect 12:P1204.
45. Dodgson KJ, Dodgson AR, Pujol C, Messer SA, Soll DR, Pfaller MA. 2005. Caspofungin resistant *C. glabrata*. Clin Microbiol Infect 11:P1158.
46. Krogh-Madsen M, Arendrup MC, Heslet L, Knudsen JD. 2006. Amphotericin B and caspofungin resistance in *Candida glabrata* isolates recovered from a critically ill patient. Clin Infect Dis 42:938–44.
47. Singh-Babak SD, Babak T, Diezmann S, Hill JA, Xie JL, Chen Y-L, Poutanen SM, Rennie RP, Heitman J, Cowen LE. 2012. Global Analysis of the Evolution and Mechanism of Echinocandin Resistance in *Candida glabrata*. PLoS Pathog 8:e1002718.
48. Durán-Valle MT, Gago S, Gómez-López A, Cuenca-Estrella M, Jiménez Díez-Canseco L, Gómez-Garcés JL, Zaragoza O. 2012. Recurrent episodes of candidemia due to *Candida glabrata* with a mutation in hot spot 1 of the *FKS2* gene developed after prolonged therapy with caspofungin. Antimicrob Agents Chemother 56:3417–9.
49. Hakki M, Staab JF, Marr KA. 2006. Emergence of a *Candida krusei* isolate with reduced susceptibility to caspofungin during therapy. Antimicrob Agents Chemother 50:2522–4.
50. Arendrup MC, Perlin DS. 2014. Echinocandin resistance: an emerging clinical problem? Curr Opin Infect Dis 27:484–492.
51. Katiyar S, Pfaller M, Edlind T. 2006. *Candida albicans* and *Candida glabrata* clinical isolates exhibiting reduced echinocandin susceptibility. Antimicrob Agents Chemother 50:2892–4.
52. Garcia-Effron G, Park S, Perlin DS. 2009. Correlating echinocandin MIC and kinetic inhibition of *FKS1* mutant glucan synthases for *Candida albicans*: implications for interpretive breakpoints. Antimicrob Agents Chemother 53:112–22.
53. Arendrup MC, Garcia-Effron G, Buzina W, Mortensen KL, Reiter N, Lundin C, Jensen HE, Lass-Flörl C, Perlin DS, Bruun B. 2009. Breakthrough *Aspergillus fumigatus* and *Candida albicans* double infection during caspofungin treatment: laboratory characteristics and implication for susceptibility testing. Antimicrob Agents Chemother 53:1185–93.
54. Balashov S V, Park S, Perlin DS. 2006. Assessing resistance to the echinocandin antifungal drug caspofungin in *Candida albicans* by profiling mutations in *FKS1*. Antimicrob Agents Chemother 50:2058–63.
55. Garcia-Effron G, Chua DJ, Tomada JR, DiPersio J, Perlin DS, Ghannoum M, Bonilla H. 2010. Novel *FKS* mutations associated with echinocandin resistance in *Candida* species. Antimicrob Agents Chemother 54:2225–7.
56. Garcia-Effron G, Lee S, Park S, Cleary JD, Perlin DS. 2009. Effect of *Candida glabrata FKS1* and *FKS2* mutations on echinocandin sensitivity and kinetics of 1,3-beta-D-glucan synthase: implication for the existing susceptibility breakpoint. Antimicrob Agents Chemother 53:3690–9.
57. Perlin DS. 2007. Resistance to echinocandin-class antifungal drugs. Drug Resist Updat 10:121–30.
58. Kahn JN, Garcia-Effron G, Hsu M-J, Park S, Marr KA, Perlin DS. 2007. Acquired echinocandin resistance in a *Candida krusei* isolate due to modification of glucan synthase. Antimicrob Agents Chemother 51:1876–8.
59. Jensen RH, Justesen US, Rewes A, Perlin DS, Arendrup MC. 2014. Echinocandin failure case due to a yet unreported *FKS1* mutation in *Candida krusei*. Antimicrob Agents Chemother 58:3550–3552.

60. Desnos-Ollivier M, Bretagne S, Raoux D, Hoinard D, Dromer F, Dannaoui E. 2008. Mutations in the *FKS1* gene in *Candida albicans*, C. *tropicalis*, and *C. krusei* correlate with elevated caspofungin MICs uncovered in AM3 medium using the method of the European Committee on Antibiotic Susceptibility Testing. Antimicrob Agents Chemother 52:3092–8.
61. Prigitano A, Esposito MC, Cogliati M, Pitzurra L, Santamaria C, Tortorano AM. 2014. Acquired echinocandin resistance in a *Candida krusei* blood isolate confirmed by mutations in the *FKS1* gene. New Microbiol 37:237–40.
62. Forastiero A, Garcia-Gil V, Rivero-Menendez O, Garcia-Rubio R, Monteiro MC, Alastruey-Izquierdo A, Jordan R, Agorio I, Mellado E. 2015. Rapid development of *Candida krusei* echinocandin resistance during caspofungin therapy. Antimicrob Agents Chemother AAC.01005–15.
63. Garcia-Effron G, Kontoyiannis DP, Lewis RE, Perlin DS. 2008. Caspofungin-resistant *Candida tropicalis* strains causing breakthrough fungemia in patients at high risk for hematologic malignancies. Antimicrob Agents Chemother 52:4181–3.
64. Jensen RH, Johansen HK, Arendrup MC. 2012. Stepwise development of homozygous S80P substitution in *FKS1*p conferring echinocandin resistance in *Candida tropicalis*. Antimicrob Agents Chemother 57:614–7.
65. Arendrup MC, Garcia-Effron G, Lass-Flörl C, Lopez AG, Rodriguez-Tudela J-L, Cuenca-Estrella M, Perlin DS. 2010. Echinocandin susceptibility testing of *Candida* species: comparison of EUCAST EDef 7.1, CLSI M27-A3, Etest, disk diffusion, and agar dilution methods with RPMI and isosensitest media. Antimicrob Agents Chemother 54:426–39.
66. Desnos-Ollivier M, Moquet O, Chouaki T, Guérin AM, Dromer F. 2011. Development of echinocandin resistance in Clavispora lusitaniae during caspofungin treatment. J Clin Microbiol 49:2304–2306.
67. Fekkar A, Meyer I, Brossas JY, Dannaoui E, Palous M, Uzunov M, Nguyen S, Leblond V, Mazier D, Datry A. 2013. Rapid emergence of echinocandin resistance during *Candida* kefyr fungemia treatment with caspofungin. Antimicrob Agents Chemother 57:2380–2.
68. Staab JF, Neofytos D, Rhee P, Jimenez-Ortigosa C, Zhang SX, Perlin DS, Marr KA. 2014. Target Enzyme Mutations Confer Differential Echinocandin Susceptibilities in *Candida* kefyr. Antimicrob Agents Chemother 58:5421–5427.
69. Garcia-Effron G, Katiyar SK, Park S, Edlind TD, Perlin DS. 2008. A naturally occurring proline-to-alanine amino acid change in *FKS1*p in *Candida* parapsilosis, *Candida* orthopsilosis, and *Candida* metapsilosis accounts for reduced echinocandin susceptibility. Antimicrob Agents Chemother 52:2305–12.
70. Mazur P, Morin N, Baginsky W, Clemas JA, Foor F, Mazur P, Morin N, Baginsky W, El-sherbeini M. 1995. Differential expression and function of two homologous subunits of yeast Differential Expression and Function of Two Homologous Subunits of Yeast 1 , 3- $^{N}_{L}$ - D -Glucan Synthase. Mol Cell Biol 15:5671–5681.
71. Johnson ME, Katiyar SK, Edlind TD. 2011. New *FKS* hot spot for acquired echinocandin resistance in Saccharomyces cerevisiae and its contribution to intrinsic resistance of Scedosporium species. Antimicrob Agents Chemother 55:3774–81.
72. Ben-Ami R, Garcia-Effron G, Lewis RE, Gamarra S, Leventakos K, Perlin DS, Kontoyiannis DP. 2011. Fitness and Virulence Costs of *Candida albicans FKS1* Hot Spot Mutations Associated With Echinocandin Resistance. J Infect Dis 204:626–35.
73. Tavanti A, Davidson AD, Johnson EM, Maiden MCJ, Shaw DJ, Gow NAR, Odds FC. 2005. Multilocus Sequence Typing for Differentiation of Strains of *Candida tropicalis*. J Clin Microbiol 43:5593–5600.
74. Axner-Elings M, Botero-Kleiven S, Jensen RH, Arendrup MC. 2011. Echinocandin susceptibility testing of *Candida* isolates collected during a 1-year period in Sweden. J Clin Microbiol 49:2516–21.
75. Niimi K, Monk BC, Hirai A, Hatakenaka K, Umeyama T, Lamping E, Maki K, Tanabe K, Kamimura T, Ikeda F, Uehara Y, Kano R, Hasegawa A, Cannon RD, Niimi M. 2010. Clinically significant micafungin resistance in *Candida albicans* involves modification of a glucan synthase catalytic subunit GSC1 (*FKS1*) allele followed by loss of heterozygosity. J Antimicrob Chemother 65:842–52.
76. Gerstein AC, Kuzmin A, Otto SP. 2014. Loss-of-heterozygosity facilitates passage through Haldane's sieve for Saccharomyces cerevisiae undergoing adaptation. Nat Commun 5:3819.
77. Shields RK, Nguyen MH, Press EG, Kwa AL, Cheng S, Du C, Clancy CJ. 2012. The Presence of an *FKS* Mutation Rather than MIC Is an Independent Risk Factor for Failure of Echinocandin Therapy among Patients with Invasive Candidiasis Due to *Candida glabrata*. Antimicrob Agents Chemother 56:4862–9.
78. Arendrup MC, Perlin DS, Jensen RH, Howard SJ, Goodwin J, Hope W. 2012. Differential In Vivo Activities of Anidulafungin, Caspofungin, and Micafungin against *Candida glabrata* Isolates with and without *FKS* Resistance Mutations. Antimicrob Agents Chemother 56:2435–2442.
79. Chapeland-Leclerc F, Hennequin C, Papon N, Noël T, Girard A, Socié G, Ribaud P, Lacroix C. 2010. Acquisition of flucytosine, azole, and caspofungin resistance in *Candida glabrata* bloodstream isolates serially obtained from a hematopoietic stem cell transplant recipient. Antimicrob Agents Chemother 54:1360–2.
80. Maubon D, Garnaud C, Calandra T, Sanglard D, Cornet M. 2014. Resistance of *Candida* spp. to antifungal drugs in the ICU: where are we now? Intensive Care Med 40:1241–55.
81. Cowen LE, Lindquist S. 2005. Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. Science 309:2185–2189.
82. Hill JA, Ammar R, Torti D, Nislow C, Cowen LE. 2013. Genetic and genomic architecture of the evolution of resistance to antifungal drug combinations. PLoS Genet 9:e1003390.
83. Morio F, Loge C, Besse B, Hennequin C, Le Pape P. 2010. Screening for amino acid substitutions in the *Candida albicans ERG11* protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. Diagn Microbiol Infect Dis 66:373–84.
84. Morschhäuser J. 2002. The genetic basis of fluconazole resistance development in *Candida albicans*. Biochim Biophys Acta 1587:240–248.
85. Kanafani ZA, Perfect JR. 2008. Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. Clin Infect Dis 46:120–8.

86. Spampinato C, Leonardi D. 2013. *Candida* infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. Biomed Res Int 2013:204237.
87. Sanglard D, Odds FC. 2002. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis 2:73–85.
88. Ghannoum MA, Rice LB. 1999. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev 12:501–17.
89. Shor E, Perlin DS. 2015. Coping with Stress and the Emergence of Multidrug Resistance in Fungi. PLOS Pathog 11:e1004668.
90. Flowers SA, Colón B, Whaley SG, Schuler MA, Rogers PD. 2015. Contribution of Clinically Derived Mutations in *ERG11* to Azole Resistance in *Candida albicans*. Antimicrob Agents Chemother 59:450–60.
91. Zhao J, Xu Y, Li C. 2013. Association of T916C (Y257H) mutation in *Candida albicans ERG11* with fluconazole resistance. Mycoses 56:315–20.
92. Oliveira Carvalho V, Okay TS, Melhem MSC, Walderez Szeszs M, del Negro GMB. 2013. The new mutation L321F in *Candida albicans ERG11* gene may be associated with fluconazole resistance. Rev Iberoam Micol 30:209–12.
93. Xiang M-J, Liu J-Y, Ni P-H, Wang S, Shi C, Wei B, Ni Y-X, Ge H-L. 2013. *ERG11* mutations associated with azole resistance in clinical isolates of *Candida albicans*. FEMS Yeast Res 13:386–93.
94. Monk BC, Tomasiak TM, Keniya M V, Huschmann FU, Tyndall JDA, O'Connell JD, Cannon RD, McDonald JG, Rodriguez A, Finer-Moore JS, Stroud RM. 2014. Architecture of a single membrane spanning cytochrome P450 suggests constraints that orient the catalytic domain relative to a bilayer. Proc Natl Acad Sci U S A 111:3865–70.
95. Debnath S, Addya S. 2014. Structural basis for heterogeneous phenotype of *ERG11* dependent Azole resistance in C.*albicans* clinical isolates. Springerplus 3:1–16.
96. Vasicek EM, Berkow EL, Flowers SA, Barker KS, Rogers PD. 2014. *UPC2* is universally essential for azole antifungal resistance in *Candida albicans*. Eukaryot Cell 13:933–46.
97. Hoot SJ, Smith AR, Brown RP, White TC. 2011. An A643V amino acid substitution in *UPC2*p contributes to azole resistance in well-characterized clinical isolates of *Candida albicans*. Antimicrob Agents Chemother 55:940–2.
98. Oliver BG, Song JL, Choiniere JH, White TC. 2007. cis-Acting elements within the *Candida albicans ERG11* promoter mediate the azole response through transcription factor *UPC2*p. Eukaryot Cell 6:2231–9.
99. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, Morschhäuser J, Rogers PD. 2012. Gain-of-function mutations in *UPC2* are a frequent cause of *ERG11* upregulation in azole-resistant clinical isolates of *Candida albicans*. Eukaryot Cell 11:1289–99.
100. Silver PM, Oliver BG, White TC. 2004. Role of *Candida albicans* transcription factor *UPC2*p in drug resistance and sterol metabolism. Eukaryot Cell 3:1391–1397.
101. Selmecki A, Forche A, Berman J. 2006. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. Science 313:367–370.
102. Coste A, Selmecki A, Forche A, Diogo D, Bougnoux ME, D'Enfert C, Berman J, Sanglard D. 2007. Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. Eukaryot Cell 6:1889–1904.
103. Basso LR, Gast CE, Mao Y, Wong B. 2010. Fluconazole transport into *Candida albicans* secretory vesicles by the membrane proteins *CDR1*p, *CDR2*p, and *MDR1*p. Eukaryot Cell 9:960–70.
104. Perlin DS. 2009. Antifungal drug resistance: do molecular methods provide a way forward? Curr Opin Infect Dis 22:568–73.
105. Sanglard D, Ischer F, Monod M, Bille J. 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of *CDR2*, a new multidrug ABC transporter gene. Microbiology 143:405–16.
106. Albertson GD, Niimi M, Cannon RD, Jenkinson HF. 1996. Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. Antimicrob Agents Chemother 40:2835–2841.
107. Chau AS, Mendrick CA, Sabatelli FJ, Loebenberg D, McNicholas PM, Loebenberg D. 2004. Application of Real-Time Quantitative PCR to Molecular Analysis of *Candida albicans* Strains Exhibiting Reduced Susceptibility to Azoles. Antimicrob Agents Chemother 48:2124–31.
108. Morio F, Pagniez F, Lacroix C, Miegeville M, Le Pape P. 2012. Amino acid substitutions in the *Candida albicans* sterol Δ5,6-desaturase (*ERG3*p) confer azole resistance: characterization of two novel mutants with impaired virulence. J Antimicrob Chemother 67:2131–8.
109. Miyazaki Y, Geber A, Miyazaki H, Falconer D, Parkinson T, Hitchcock C, Grimberg B, Nyswaner K, Bennett JE. 1999. Cloning, sequencing, expression and allelic sequence diversity of *ERG3* (C-5 sterol desaturase gene) in *Candida albicans*. Gene 236:43–51.
110. Pierce AM, Pierce HD, Unrau AM, Oehlschlager AC. 1978. Lipid composition and polyene antibiotic resistance of *Candida albicans* mutants. Can J Biochem 56:135–142.
111. Ellis D. 2002. Amphotericin B: spectrum and resistance. J Antimicrob Chemother 49:7–10.
112. Broughton MC, Bard M, Lees ND. 1991. Polyene resistance in ergosterol producing strains of *Candida albicans*. Mycoses 34:75–83.
113. Canuto MM, Rodero FG. 2002. Antifungal drug resistance Antifungal drug resistance to azoles and polyenes. Lancet 2:550–563.
114. Nolte FS, Parkinson T, Falconer DJ, Dix S, Williams J, Gilmore C, Geller R, Wingard JR. 1997. Isolation and characterization of fluconazole- and amphotericin B-resistant *Candida albicans* from blood of two patients with leukemia. Antimicrob Agents Chemother 41:196–199.
115. Kelly SL, Lamb DC, Kelly DE, Loeffler J, Einsele H. 1996. Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients. Lancet 348:5–6.
116. Kelly S., Lamb D., Kelly D., Manning N., Loeffler J, Hebart H, Schumacher U, Einsele H. 1997. Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol Δ5,6-desaturation. FEBS Lett 400:80–82.
117. Young LY, Hull CM, Heitman J. 2003. Disruption of Ergosterol Biosynthesis Confers Resistance to Amphotericin B in *Candida* lusitaniae. Antimicrob Agents Chemother 47:2717–2724.

118. Martel CM, Parker JE, Bader O, Weig M, Gross U, Warrilow AGS, Kelly DE, Kelly SL. 2010. A clinical isolate of *Candida albicans* with mutations in *ERG11* (encoding sterol 14alpha-demethylase) and *ERG5* (encoding C22 desaturase) is cross resistant to azoles and amphotericin B. Antimicrob Agents Chemother 54:3578–83.
119. Jensen RH, Astvad KMT, Silva LV, Sanglard D, Jørgensen R, Nielsen KF, Mathiasen EG, Doroudian G, Perlin DS, Arendrup MC. 2015. Stepwise emergence of azole, echinocandin and amphotericin B multidrug resistance in vivo in *Candida albicans* orchestrated by multiple genetic alterations. J Antimicrob Chemother 70:2551–2555.
120. Bougnoux M-E, Morand S, Enfert C, D'Enfert C. 2002. Usefulness of Multilocus Sequence Typing for Characterization of Clinical Isolates of *Candida albicans*. J Clin Microbiol 40:1290–7.
121. Tavanti A, Gow NAR, Senesi S, Maiden MCJ, Odds FC. 2003. Optimization and validation of multilocus sequence typing for *Candida albicans*. J Clin Microbiol 41:3765–76.
122. Lohberger A, Coste AT, Sanglard D. 2014. Distinct roles of *Candida albicans* drug resistance transcription factors *TAC1*, *MRR1*, and *UPC2* in virulence. Eukaryot Cell 13:127–42.
123. Pierson CA, Eckstein J, Barbuch R, Bard M. 2004. Ergosterol gene expression in wild-type and ergosterol-deficient mutants of *Candida albicans*. Med Mycol 42:385–389.
124. Kelley LA, Sternberg MJE. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc 4:363–71.
125. Morio F, Pagniez F, Besse M, Gay-andrieu F, Miegeville M, Le Pape P. 2013. Deciphering azole resistance mechanisms with a focus on transcription factor-encoding genes *TAC1*, *MRR1* and *UPC2* in a set of fluconazole-resistant clinical isolates of *Candida albicans*. Int J Antimicrob Agents 42:410–5.
126. Hull CM, Bader O, Parker JE, Weig M, Gross U, Warrilow AGS, Kelly DE, Kelly SL. 2012. Two Clinical Isolates of *Candida glabrata* Exhibiting Reduced Sensitivity to Amphotericin B Both Harbor Mutations in *ERG2*. Antimicrob Agents Chemother 56:6417–6421.
127. Kumar R, Shukla PK. 2010. Amphotericin B resistance leads to enhanced proteinase and phospholipase activity and reduced germ tube formation in *Candida albicans*. Fungal Biol 114:189–97.
128. Lackner M, Tscherner M, Schaller M, Kuchler K, Mair C, Sartori B, Istel F, Arendrup MC. 2014. Positions and Numbers of *FKS* Mutations in *Candida albicans* Selectively Influence In Vitro and In Vivo Susceptibilities to 58:3626–3635.
129. Scorzoni L, de Lucas MP, Mesa-Arango AC, Fusco-Almeida AM, Lozano E, Cuenca-Estrella M, Mendes-Giannini MJ, Zaragoza O. 2013. Antifungal efficacy during *Candida krusei* infection in non-conventional models correlates with the yeast in vitro susceptibility profile. PLoS One 8:e60047.
130. Cho E-J, Shin JH, Kim SH, Kim H-K, Park JS, Sung H, Kim M-N, Im HJ. 2015. Emergence of multiple resistance profiles involving azoles, echinocandins and amphotericin B in *Candida glabrata* isolates from a neutropenia patient with prolonged fungaemia. J Antimicrob Chemother 70:1268–70.
131. Garnaud C, Botterel F, Sertour N, Bougnoux M-E, Dannaoui E, Larrat S, Hennequin C, Guinea J, Cornet M, Maubon D. 2015. Next-generation sequencing offers new insights into the resistance of *Candida* spp. to echinocandins and azoles. J Antimicrob Chemother 70:2556–2565.
132. Blanchard E, Lortholary O, Boukris-Sitbon K, Desnos-Ollivier M, Dromer F, Guillemot D. 2011. Prior caspofungin exposure in patients with hematological malignancies is a risk factor for subsequent fungemia due to decreased susceptibility in *Candida* spp.: a case-control study in Paris, France. Antimicrob Agents Chemother 55:5358–61.
133. Lortholary O, Desnos-Ollivier M, Sitbon K, Fontanet A, Bretagne S, Dromer F. 2011. Recent exposure to caspofungin or fluconazole influences the epidemiology of candidemia: a prospective multicenter study involving 2,441 patients. Antimicrob Agents Chemother 55:532–8.
134. Jensen RH, Johansen HK, Søes LM, Lemming LE, Rosenvinge FS, Nielsen L, Olesen B, Kristensen L, Leitz C, Dzajic E, Kjaeldgaard P, Astvad KMT, Arendrup MC. 2015. Post treatment antifungal resistance among colonizing *Candida* isolates in candidemia patients: results from a systematic multicentre study. Submitted.
135. Lockhart SR, Fritch JJ, Meier AS, Schroppel K, Srikantha T, Galask R, Soll DR. 1995. Colonizing populations of *Candida albicans* are clonal in origin but undergo microevolution through C1 fragment reorganization as demonstrated by DNA fingerprinting and C1 sequencing. J Clin Microbiol 33:1501–1509.
136. Mann PA, McNicholas PM, Chau AS, Patel R, Mendrick C, Ullmann AJ, Cornely OA, Patino H, Black TA. 2009. Impact of antifungal prophylaxis on colonization and azole susceptibility of *Candida* species. Antimicrob Agents Chemother 53:5026–5034.
137. Gammelsrud KW, Lindstad BL, Gaustad P, Ingebretsen A, Høiby EA, Brandtzaeg P, Sandven P. 2012. Multilocus sequence typing of serial *Candida albicans* isolates from children with cancer, children with cystic fibrosis and healthy controls. Med Mycol 50:619–26.
138. Brillowska-Dabrowska A, Bergmann O, Jensen IM, Jarløv JO, Arendrup MC. 2010. Typing of *Candida* isolates from patients with invasive infection and concomitant colonization. Scand J Infect Dis 42:109–13.
139. Lau AF, Kabir M, Chen SC-A, Playford EG, Marriott DJ, Jones M, Lipman J, McBryde E, Gottlieb T, Cheung W, Seppelt I, Iredell J, Sorrell TC. 2015. *Candida* colonization as a risk marker for invasive candidiasis in mixed medical-surgical intensive care units: development and evaluation of a simple, standard protocol. J Clin Microbiol 53:1324–30.
140. Odds FC, Jacobsen MD. 2008. Multilocus sequence typing of pathogenic *Candida* species. Eukaryot Cell 7:1075–84.
141. McManus BA, Coleman DC, Moran G, Pinjon E, Diogo D, Bougnoux M-E, Borecká-Melkusova S, Bujdákova H, Murphy P, D'Enfert C, Sullivan DJ. 2008. Multilocus sequence typing reveals that the population structure of *Candida* dubliniensis is significantly less divergent than that of *Candida albicans*. J Clin Microbiol 46:652–64.
142. Dodgson AR, Pujol C, Denning DW, Soll DR, Fox AJ. 2003. Multilocus Sequence Typing of *Candida glabrata* Reveals Geographically Enriched Clades. J Clin Microbiol 41:5709–5717.
143. Jacobsen MD, Gow NAR, Maiden MCJ, Shaw DJ, Odds FC. 2007. Strain typing and determination of population structure of *Candida krusei* by multilocus sequence typing. J Clin Microbiol 45:317–23.
144. Diab-Elschahawi M, Forstner C, Hagen F, Meis JF, Lassnig AM, Presterl E, Klaassen CHW. 2012. Microsatellite genotyp-

ing clarified conspicuous accumulation of *Candida* parapsilosis at a cardio-thoracic surgery intensive care unit. J Clin Microbiol 50:3422–6.
145. Ayoub MJ, Legras JL, Saliba R, Gaillardin C. 2006. Application of Multi Locus Sequence Typing to the analysis of the biodiversity of indigenous Saccharomyces cerevisiae wine yeasts from Lebanon. J Appl Microbiol 100:699–711.
146. Arendrup MC, Bruun B, Christensen JJ, Fuursted K, Johansen HK, Kjaeldgaard P, Knudsen JD, Kristensen L, Møller J, Nielsen L, Rosenvinge FS, Røder B, Schønheyder HC, Thomsen MK, Truberg K. 2011. National surveillance of fungemia in Denmark (2004 to 2009). J Clin Microbiol 49:325–34.
147. Alexander BD, Johnson MD, Pfeiffer CD, Jiménez-Ortigosa C, Catania J, Booker R, Castanheira M, Messer SA, Perlin DS, Pfaller MA. 2013. Increasing echinocandin resistance in *Candida glabrata*: clinical failure correlates with presence of *FKS* mutations and elevated minimum inhibitory concentrations. Clin Infect Dis 56:1724–32.
148. Farmakiotis D, Tarrand JJ, Kontoyiannis DP. 2014. Drug-resistant *Candida glabrata* infection in cancer patients. Emerg Infect Dis 20:1833–40.
149. Panackal AA, Gribskov JL, Staab JF, Kirby KA, Rinaldi M, Marr KA. 2006. Clinical Significance of Azole Antifungal Drug Cross-Resistance in *Candida glabrata*. J Clin Microbiol 44:1740–1743.
150. Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J. 2012. *Candida glabrata*, *Candida* parapsilosis and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. FEMS Microbiol Rev 36:288–305.
151. Rodrigues CF, Silva S, Henriques M. 2014. *Candida glabrata*: a review of its features and resistance. Eur J Clin Microbiol Infect Dis 33:673–88.
152. Ostrosky-Zeichner L. 2013. *Candida glabrata* and *FKS* mutations: witnessing the emergence of the true multidrug-resistant *Candida*. Clin Infect Dis 56:1733–4.
153. Van Asbeck EC, Huang Y-C, Markham AN, Clemons K V, Stevens DA. 2007. *Candida* parapsilosis fungemia in neonates: genotyping results suggest healthcare workers hands as source, and review of published studies. Mycopathologia 164:287–93.
154. Reiss E, Lasker BA, Lott TJ, Bendel CM, Kaufman DA, Hazen KC, Wade KC, Mcgowan KL, Lockhart SR. 2012. Genotyping of *Candida* parapsilosis from three neonatal intensive care units (NICUs) using a panel of five multilocus microsatellite markers: Broad genetic diversity and a cluster of related strains in one NICU. Infect Genet Evol 12:1654–1660.
155. Brillowska-Dabrowska A, Schön T, Pannanusorn S, Lönnbro N, Bernhoff L, Bonnedal J, Haggstrom J, Wistedt A, Fernandez V, Arendrup MC. 2009. A nosocomial outbreak of *Candida* parapsilosis in southern Sweden verified by genotyping. Scand J Infect Dis 41:135–42.
156. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 39:309–17.
157. Crump KR, Edlind TD. 2004. Agricultural Fungicides May Select for Azole Antifungal Resistance in Pathogenic *Candida*44th Interscience Conference in Antimicrobial Agents and Chemotherapy. Abstract M1684.
158. Müller FMC, Staudigel A, Salvenmoser S, Tredup A, Miltenberger R, Herrmann J V. 2007. Cross-resistance to medical and agricultural azole drugs in yeasts from the oropharynx of human immunodeficiency virus patients and from environmental Bavarian vine grapes. Antimicrob Agents Chemother 51:3014–3016.
159. Gammelsrud KW, Sandven P, Høiby EA, Sandvik L, Brandtzaeg P, Gaustad P. 2011. Colonization by *Candida* in children with cancer, children with cystic fibrosis, and healthy controls. Clin Microbiol Infect 17:1875–81.
160. Denning DW, Anderson MJ, Turner G, Latgé J, Bennett JW. 2002. Sequencing the *Aspergillus fumigatus* genome. Lancet Infect Dis 2:251–253.
161. Denning DW, Park S, Lass-Florl C, Fraczek MG, Kirwan M, Gore R, Smith J, Bueid A, Moore CB, Bowyer P, Perlin DS. 2011. High-frequency triazole resistance found In nonculturable *Aspergillus fumigatus* from lungs of patients with chronic fungal disease. Clin Infect Dis 52:1123–9.
162. Kosmidis C, Denning DW. 2015. The clinical spectrum of pulmonary aspergillosis. Thorax 70:270–277.
163. Verweij PE, Ananda-Rajah M, Andes D, Arendrup MC, Brüggemann RJ, Chowdhary A, Cornely OA, Denning DW, Groll AH, Izumikawa K, Kullberg BJ, Lagrou K, Maertens J, Meis JF, Newton P, Page I, Seyedmousavi S, Sheppard DC, Viscoli C, Warris A, Donnelly JP. 2015. International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. Drug Resist Updat 21-22:30–40.
164. Bennett JW. 2009. *Aspergillus*: a primer for the novice. Med Mycol 47:S5–S12.
165. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, Pappas PG, Maertens J, Lortholary O, Kauffman CA, Denning DW, Patterson TF, Maschmeyer G, Bille J, Dismukes WE, Herbrecht R, Hope WW, Kibbler CC, Kullberg BJ, Marr KA, Muñoz P, Odds FC, Perfect JR, Restrepo A, Ruhnke M, Segal BH, Sobel JD, Sorrell TC, Viscoli C, Wingard JR, Zaoutis T, Bennett JE. 2008. Revised Definitions of Invasive Fungal Disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) C. Clin Infect Dis 46:1813–1821.
166. Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann J-W, Kern W V, Marr KA, Ribaud P, Lortholary O, Sylvester R, Rubin RH, Wingard JR, Stark P, Durand C, Caillot D, Thiel E, Chandrasekar PH, Hodges MR, Schlamm HT, Troke PF, de Pauw B. 2002. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med 347:408–15.
167. Brown G, Denning D. 2012. Hidden killers: human fungal infections. Sci Transl Med 4:165rv13.
168. Bowyer P, Moore CB, Rautemaa R, Denning DW, Richardson MD. 2011. Azole antifungal resistance today: Focus on *Aspergillus*. Curr Infect Dis Rep 13:485–491.
169. Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IS, Denning DW. 2002. Increased expression of a novel *Aspergillus fumigatus* ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet Biol 36:199–206.
170. Camps SMT, Dutilh BE, Arendrup MC, Rijs AJMM, Snelders E, Huynen MA, Verweij PE, Melchers WJG. 2012. Discovery of a HapE mutation that causes azole resistance in *Aspergil-*

lus fumigatus through whole genome sequencing and sexual crossing. PLoS One 7:e50034.
171. Verweij PE, Snelders E, Kema GHJ, Mellado E, Melchers WJG. 2009. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? Lancet Infect Dis 9:789–95.
172. Balajee SA, de Valk HA, Lasker BA, Meis JFGM, Klaassen CHW. 2008. Utility of a microsatellite assay for identifying clonally related outbreak isolates of *Aspergillus fumigatus*. J Microbiol Methods 73:252–6.
173. Peláez T, Muñoz P, Guinea J, Valerio M, Giannella M, Klaassen CHW, Bouza E. 2012. Outbreak of invasive aspergillosis after major heart surgery caused by spores in the air of the intensive care unit. Clin Infect Dis 54:e24–31.
174. Van der Linden JWM, Camps SMT, Kampinga GA, Arends JPA, Debets-Ossenkopp YJ, Haas PJA, Rijnders BJA, Kuijper EJ, Tiel FH Van, Varga J, Karawajczyk A, Zoll J, Melchers WJG, Verweij PE. 2013. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. Clin Infect Dis 57:513–20.
175. ECDC. 2013. European Centre for Disease Prevention and Control. Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species. Stockholm, ECDC.
176. Van der Linden JWM, Snelders E, Kampinga GA, Rijnders BJA, Mattsson E, Debets-ossenkopp YJ, Kuijper EJ, Van Tiel FH, Melchers WJG, Verweij PE. 2011. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007-2009. Emerg Infect Dis 17:1846–54.
177. Vermeulen E, Lagrou K, Verweij PE. 2013. Azole resistance in *Aspergillus fumigatus*. Curr Opin Infect Dis 26:493–500.
178. Camps SMT, van der Linden JWM, Li Y, Kuijper EJ, van Dissel JT, Verweij PE, Melchers WJG. 2012. Rapid induction of multiple resistance mechanisms in *Aspergillus fumigatus* during azole therapy: a case study and review of the literature. Antimicrob Agents Chemother 56:10–6.
179. Chowdhary A, Sharma C, Kathuria S, Hagen F, Meis JF. 2014. Azole-resistant *Aspergillus fumigatus* with the environmental TR_{46}/Y121F/T289A mutation in India. J Antimicrob Chemother 69:555–7.
180. Camps SMT, Rijs AJMM, Klaassen CHW, Meis JF, O'Gorman CM, Dyer PS, Melchers WJG, Verweij PE. 2012. Molecular epidemiology of *Aspergillus fumigatus* isolates harboring the TR_{34}/L98H azole resistance mechanism. J Clin Microbiol 50:2674–80.
181. Snelders E, Camps SMT, Karawajczyk A, Schaftenaar G, Kema GHJ, van der Lee HA, Klaassen CH, Melchers WJG, Verweij PE. 2012. Triazole Fungicides Can Induce Cross-Resistance to Medical Triazoles in *Aspergillus fumigatus*. PLoS One 7:e31801.
182. Stensvold CR, Jørgensen LN, Arendrup MC. 2012. Azole-Resistant Invasive Aspergillosis: Relationship to Agriculture. Curr Fungal Infect Rep 6:178–191.
183. Mortensen KL, Jensen RH, Johansen HK, Skov M, Pressler T, Howard SJ, Leatherbarrow H, Mellado E, Arendrup MC. 2011. *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. J Clin Microbiol 49:2243–51.
184. Astvad KMT, Jensen RH, Hassan TM, Mathiasen EG, Thomsen GM, Pedersen UG, Christensen M, Hilberg O, Arendrup MC. 2014. First Detection of TR_{46}/Y121F/T289A and TR_{34}/L98H Alterations in *Aspergillus fumigatus* Isolates from Azole-Naive Patients in Denmark despite Negative Findings in the Environment. Antimicrob Agents Chemother 58:5096–101.
185. Mortensen KL, Mellado E, Lass-Flörl C, Rodriguez-Tudela JL, Johansen HK, Arendrup MC. 2010. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. Antimicrob Agents Chemother 54:4545–9.
186. Fischer J, Van Koningsbruggen-Rietschel S, Rietschel E, Vehreschild MJGT, Wisplinghoff H, Krönke M, Hamprecht A. 2014. Prevalence and molecular characterization of azole resistance in *Aspergillus* spp. isolates from German cystic fibrosis patients. J Antimicrob Chemother 69:1533–1536.
187. Vermeulen E, Maertens J, Schoemans H, Lagrou K. 2012. Azole-resistant *Aspergillus fumigatus* due to TR_{46}/Y121F/T289A mutation emerging in Belgium, July 2012. Euro Surveill 17:1–3.
188. Mellado E, De La Camara R, Buendía B, Rodriguez-Tudela JL, Cuenca-Estrella M. 2013. Breakthrough pulmonary *Aspergillus fumigatus* infection with multiple triazole resistance in a Spanish patient with chronic myeloid leukemia. Rev Iberoam Micol 30:64–8.
189. Chowdhary A, Kathuria S, Xu J, Sharma C, Sundar G, Singh PK, Gaur SN, Hagen F, Klaassen CH, Meis JF. 2012. Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR_{34}/L98H mutations in the *CYP51A* gene in India. PLoS One 7:e52871.
190. Chowdhary A, Sharma C, van den Boom M, Yntema JB, Hagen F, Verweij PE, Meis JF. 2014. Multi-azole-resistant *Aspergillus fumigatus* in the environment in Tanzania. J Antimicrob Chemother 69:2979–2983.
191. Lockhart SR, Frade JP, Etienne KA, Pfaller MA, Diekema DJ, Balajee SA. 2011. Azole resistance in *Aspergillus fumigatus* isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the *CYP51A* gene. Antimicrob Agents Chemother 55:4465–8.
192. Ahmad S, Khan Z, Hagen F, Meis JF. 2014. Occurrence of triazole-resistant *Aspergillus fumigatus* with TR_{34}/L98H mutations in outdoor and hospital environment in Kuwait. Environ Res 133:20–6.
193. Badali H, Vaezi A, Haghani I, Yazdanparast SA, Hedayati MT, Mousavi B, Ansari S, Hagen F, Meis JF, Chowdhary A. 2013. Environmental study of azole-resistant *Aspergillus fumigatus* with TR_{34}/L98H mutations in the *CYP51A* gene in Iran. Mycoses 56:659–63.
194. Seyedmousavi S, Hashemi SJ, Zibafar E, Zoll J, Hedayati MT, Mouton JW, Melchers WJG, Verweij PE. 2013. Azole-Resistant *Aspergillus fumigatus* , Iran. Emerg Infect Dis 19:3–5.
195. Özmerdiven GE, Ak S, Ener B, Ağca H, Cilo BD, Tunca B, Akalın H. 2015. First determination of azole resistance in *Aspergillus fumigatus* strains carrying the TR_{34}/L98H mutations in Turkey. J Infect Chemother 21:581–586.
196. Prigitano A, Venier V, Cogliati M, De Lorenzis G, Esposto MC, Tortorano AM. 2014. Azole-resistant *Aspergillus fumigatus*

in the environment of Northern Italy, May 2011 to June 2012. Eurosurveillance 19:1–7.
197. Warris A, Klaassen CHW, Meis JFGM, de Ruiter MT, de Valk HA, Abrahamsen TG, Gaustad P, Verweij PE. 2003. Molecular Epidemiology of *Aspergillus fumigatus* Isolates Recovered from Water, Air, and Patients Shows Two Clusters of Genetically Distinct Strains. J Clin Microbiol 41:4101–4106.
198. Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fischer MC, Pasqualotto AC, Laverdiere M, Arendrup MC, Perlin DS, Denning DW. 2009. Frequency and Evolution of Azole Resistance in *Aspergillus fumigatus* Associated with Treatment Failure. Emerg Infect Dis 15:1068–1076.
199. Montesinos I, Dodemont M, Lagrou K, Jacobs F, Etienne I, Denis O. 2014. New case of azole-resistant *Aspergillus fumigatus* due to TR_{46}/Y121F/T289A mutation in Belgium. J Antimicrob Chemother 69:3439–3440.
200. Pelaez T, Monteiro MC, Garcia-Rubio R, Bouza E, Gomez-Lopez A, Mellado E. 2015. First detection of *Aspergillus fumigatus* azole-resistant strain due to *CYP51A* TR_{46}/Y121F/T289A in an azole-naive patient in Spain. New Microbes New Infect 6:33–34.
201. Chen Y, Wang H, Lu Z, Li P, Zhang Q, Jia T, Zhao J, Tian S, Han X, Chen F, Zhang C, Jia X, Huang L, Qu F, Han L. 2015. Emergence of TR_{46}/Y121F/T289A in *Aspergillus fumigatus* Isolate from a Chinese Patient. Antimicrob Agents Chemother AAC.00887–15.
202. Bromley MJ, van Muijlwijk G, Fraczek MG, Robson G, Verweij PE, Denning DW, Bowyer P. 2014. Occurrence of azole-resistant species of *Aspergillus* in the UK environment. J Glob Antimicrob Resist 2:276–279.
203. Bader O, Tünnermann J, Dudakova A, Tangwattanachuleeporn M, Weig M, Groß U. 2015. Environmental Isolates of Azole-Resistant *Aspergillus fumigatus* in Germany. Antimicrob Agents Chemother 59:4356–4359.
204. Liu M, Zeng R, Zhang L, Li D, Lv G, Shen Y, Zheng H, Zhang Q, Zhao J, Zheng N, Liu W. 2015. Multiple *CYP51A* -Based Mechanisms Identified in Azole-Resistant Isolates of *Aspergillus fumigatus* from China. Antimicrob Agents Chemother 59:4321–4325.
205. Kidd SE, Goeman E, Meis JF, Slavin MA, Verweij PE. 2015. Multi-triazole-resistant *Aspergillus fumigatus* infections in Australia. Mycoses 58:350–5.
206. Pham CD, Reiss E, Hagen F, Meis JF, Lockhart SR. 2014. Passive surveillance for azole-resistant *Aspergillus fumigatus*, United States, 2011-2013. Emerg Infect Dis 20:1498–503.
207. Garcia-Rodriguez J, Lindner J, Sanders C, Fan H, Sutton DA, Fothergill AW. 2015. Evaluation of *CYP51A* Mechanisms of Azole Resistance in *Aspergillus fumigatus* Isolates from the United States, p. P009. In Trends in Medical Mycology.
208. Alvarez A, Lavergne R-A, Morio F. 2015. First description of fungicide-driven alteratinos in azole-resistant *Aspergillus fumigatus* in Colombia, South America, p. P019. In Trends in Medical Mycology.
209. Chowdhary A, Sharma C, Kathuria S, Hagen F, Meis JF. 2015. Prevalence and mechanism of triazole resistance in *Aspergillus fumigatus* in a referral chest hospital in Delhi, India and an update of the situation in Asia. Front Microbiol 06:1–10.
210. Nawrot U, Brillowska-Dabrowska A, Mroczynska M, Wlodarczyk K, Ussowicz M, Zdziarski P, Arendrup MC, Brillowska-Dabrowska A. 2015. Detection of clinical *Aspergillus fumigatus* isolates resistant to triazoles, p. P006. In Trends in Medical Mycology.
211. Wu C-J, Wang H-C, Lee J-C, Lo H-J, Dai C-T, Chou P-H, Ko W-C, Chen Y-C. 2015. Azole-resistant *Aspergillus fumigatus* isolates carrying TR 34 /L98H mutations in Taiwan. Mycoses 58:544–549.
212. Alanio A, Sitterlé E, Liance M, Farrugia C, Foulet F, Botterel F, Hicheri Y, Cordonnier C, Costa J-MM, Bretagne S. 2011. Low prevalence of resistance to azoles in *Aspergillus fumigatus* in a French cohort of patients treated for haematological malignancies. J Antimicrob Chemother 66:371–4.
213. Hodiamont CJ, Dolman KM, Ten Berge IJM, Melchers WJG, Verweij PE, Pajkrt D. 2009. Multiple-azole-resistant *Aspergillus fumigatus* osteomyelitis in a patient with chronic granulomatous disease successfully treated with long-term oral posaconazole and surgery. Med Mycol 47:217–20.
214. De Valk HA, Meis JFGM, Curfs IM, Muehlethaler K, Mouton JW, Klaassen CHW. 2005. Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of *Aspergillus fumigatus* isolates. J Clin Microbiol 43:4112–20.
215. Verweij PE, Howard SJ, Melchers WJG, Denning DW. 2009. Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints. Drug Resist Updat 12:141–7.
216. Snelders E, Karawajczyk A, Verhoeven RJA, Venselaar H, Schaftenaar G, Verweij PE, Melchers WJG. 2011. The structure-function relationship of the *Aspergillus fumigatus CYP51A* L98H conversion by site-directed mutagenesis: The mechanism of L98H azole resistance. Fungal Genet Biol 48:1062–1070.
217. Lescar J, Meyer I, Akshita K, Srinivasaraghavan K, Verma C, Palous M, Mazier D, Datry A, Fekkar A. 2014. *Aspergillus fumigatus* harbouring the sole Y121F mutation shows decreased susceptibility to voriconazole but maintained susceptibility to itraconazole and posaconazole. J Antimicrob Chemother 69:3244–7.
218. Tashiro M, Izumikawa K, Minematsu A, Hirano K, Iwanaga N, Ide S, Mihara T, Hosogaya N, Takazono T, Morinaga Y, Nakamura S, Kurihara S, Imamura Y, Miyazaki T, Nishino T, Tsukamoto M, Kakeya H, Yamamoto Y, Yanagihara K, Yasuoka A, Tashiro T, Kohno S. 2012. Antifungal Susceptibilities of *Aspergillus fumigatus* Clinical Isolates Obtained in Nagasaki, Japan. Antimicrob Agents Chemother 56:584–7.
219. Snelders E, Camps SMT, Karawajczyk A, Rijs AJMM, Zoll J, Verweij PE, Melchers WJG. 2015. Genotype–phenotype complexity of the TR_{46}/Y121F/T289A *CYP51A* azole resistance mechanism in *Aspergillus fumigatus*. Fungal Genet Biol 82:129–135.
220. Pelaez T, Gijon P, Bunsow E, Bouza E, Sanchez-Yebra W, Valerio M, Gama B, Cuenca-Estrella M, Mellado E. 2012. Resistance to Voriconazole Due to a G448S Substitution in *Aspergillus fumigatus* in a Patient with Cerebral Aspergillosis. J Clin Microbiol 50:2531–2534.
221. Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE, Melchers WJG. 2010. Azole resistance profile of amino acid changes in *Aspergillus fumigatus CYP51A* based on protein homology modeling. Antimicrob Agents Chemother 54:2425–30.

222. Fraczek MG, Bromley M, Bowyer P. 2011. An improved model of the *Aspergillus fumigatus CYP51A* protein. Antimicrob Agents Chemother 55:2483–6.
223. Jensen RH, Hagen F, Astvad KMT, Tyron A, Meis JF, Arendrup MC. 2015. Azole resistant *Aspergillus fumigatus* is a persistent threat in Denmark: results from a laboratory based study and focus on genotyping data.In preparation.
224. Arendrup MC, Jensen RH, Grif K, Skov M, Pressler T, Johansen HK, Lass-Flörl C. 2012. In vivo emergence of *Aspergillus* terreus with reduced azole susceptibility and a *CYP51A* M217I Alteration. J Infect Dis 206:981–985.
225. Weber DJ, Peppercorn A, Miller MB, Sickbert-Benett E, Rutala W a. 2009. Preventing healthcare-associated *Aspergillus* infections: review of recent CDC/HICPAC recommendations. Med Mycol 47:S199–S209.
226. Van Waeyenberghe L, Pasmans F, Beernaert LA, Haesebrouck F, Vercammen F, Verstappen F, Dorrestein GM, Klaassen CHW, Martel A. 2011. Microsatellite typing of avian clinical and environmental isolates of *Aspergillus fumigatus*. Avian Pathol 40:73–7.
227. Escribano P, Recio S, Peláez T, Bouza E, Guinea J. 2011. *Aspergillus fumigatus* strains with mutations in the *CYP51A* gene do not always show phenotypic resistance to itraconazole, voriconazole, or posaconazole. Antimicrob Agents Chemother 55:2460–2.
228. Klaassen CHW, Gibbons JG, Fedorova ND, Meis JF, Rokas A. 2012. Evidence for genetic differentiation and variable recombination rates among Dutch populations of the opportunistic human pathogen *Aspergillus fumigatus*. Mol Ecol 21:57–70.
229. Abdolrasouli A, Rhodes J, Beale MA, Hagen F, Rogers TR, Chowdhary A, Meis JF, Armstrong-james D, Fisher MC. 2015. Genomic Context of Azole Resistance Mutations in *Aspergillus fumigatus* Determined Using Whole-Genome Sequencing. MBio 6:1–11.
230. Lewis HC, Mølbak K, Reese C, Aarestrup FM, Selchau M, Sørum M, Skov RL. 2008. Pigs as source of methicillin-resistant Staphylococcus aureus CC398 infections in humans, Denmark. Emerg Infect Dis 14:1383–9.
231. Price LB, Stegger M, Hasman H, Aziz M, Larsen J, Andersen S, Pearson T. 2012. Adaptation and emergence of Staphylococcus aureus CC39: Host adaptation and emergence of methicillin resistance in livestock. MBio 3:1–6.
232. Finkel JS, Mitchell AP. 2011. Genetic control of *Candida albicans* biofilm development. Nat Rev Microbiol 9:109–18.
233. Finkel JS, Xu W, Huang D, Hill EM, Desai J V, Woolford C a, Nett JE, Taff H, Norice CT, Andes DR, Lanni F, Mitchell AP. 2012. Portrait of *Candida albicans* adherence regulators. PLoS Pathog 8:e1002525.
234. Steier Z, Vermitsky J-P, Toner G, Gygax SE, Edlind T, Katiyar S. 2013. Flucytosine antagonism of azole activity versus *Candida glabrata*: role of transcription factor Pdr1 and multidrug transporter *CDR1*. Antimicrob Agents Chemother 57:5543–7.
235. Edlind TD, Katiyar SK. 2010. Mutational analysis of flucytosine resistance in *Candida glabrata*. Antimicrob Agents Chemother 54:4733–8.
236. Zhao Y, Stensvold CR, Perlin DS, Arendrup MC. 2013. Azole resistance in *Aspergillus fumigatus* from bronchoalveolar lavage fluid samples of patients with chronic diseases. J Antimicrob Chemother 68:1497–504.
237. Wiederhold NP, Grabinski JL, Garcia-Effron G, Perlin DS, Lee SA. 2008. Pyrosequencing to detect mutations in *FKS1* that confer reduced echinocandin susceptibility in *Candida albicans*. Antimicrob Agents Chemother 52:4145–8.
238. Dudiuk C, Gamarra S, Jimenez-Ortigosa C, Leonardelli F, Macedo D, Perlin DS, Garcia-Effron G. 2015. Quick Detection of *FKS1* Mutations Responsible for Clinical Echinocandin Resistance in *Candida albicans*. J Clin Microbiol 53:2037–2041.
239. Mukherjee PK, Sendid B, Hoarau G, Colombel J-F, Poulain D, Ghannoum MA. 2014. Mycobiota in gastrointestinal diseases. Nat Rev Gastroenterol Hepatol 12:77–87.
240. Marchesi JR, Adams DH, Fava F, Hermes GDA, Hirschfield GM, Hold G, Quraishi MN, Kinross J, Smidt H, Tuohy KM, Thomas L V, Zoetendal EG, Hart A. 2015. The gut microbiota and host health: a new clinical frontier. Gut 1–10.
241. Van Ingen J, van der Lee HA, Rijs TAJ, Zoll J, Leenstra T, Melchers WJG, Verweij PE. 2015. Azole, polyene and echinocandin MIC distributions for wild-type, TR_{34}/L98H and TR_{46}/Y121F/T289A *Aspergillus fumigatus* isolates in the Netherlands. J Antimicrob Chemother 70:178–81.