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Aspergillus fumigatus and aspergillosis: From basics to clinics

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Abstract: The airborne fungus *Aspergillus fumigatus* poses a serious health threat to humans by causing numerous invasive infections and a notable mortality in humans, especially in immunocompromised patients. Mould-active azoles are the frontline therapeutics employed to treat aspergillosis. The global emergence of azole-resistant *A. fumigatus* isolates in clinic and environment, however, notoriously limits the therapeutic options of mould-active antifungals and potentially can be attributed to a mortality rate reaching up to 100 %. Although specific mutations in *CYP51A* are the main cause of azole resistance, there is a new wave of azole-resistant isolates with wild-type *CYP51A* genotype challenging the efficacy of the current diagnostic tools. Therefore, applications of whole-genome sequencing are increasingly gaining popularity to overcome such challenges. Prominent echinocandin tolerance, as well as liver and kidney toxicity posed by amphotericin B, necessitate a continuous quest for novel antifungal drugs to combat emerging azole-resistant *A. fumigatus* isolates. Animal models and the tools used for genetic engineering require further refinement to facilitate a better understanding about the resistance mechanisms, virulence, and immune reactions orchestrated against *A. fumigatus*. This review paper comprehensively discusses the current clinical challenges caused by *A. fumigatus* and provides insights on how to address them.

Key words: *Aspergillus fumigatus*, Azole-resistance, Drug-resistance mechanism, Invasive aspergillosis.

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INTRODUCTION

Species within the genus *Aspergillus* have been long exploited as an invaluable biotechnological resource to produce pharmaceuticals, food and food ingredients, and enzymes (reviewed in Meyer *et al.* 2011). Among species within this genus, *Aspergillus fumigatus* is the most ubiquitous fungal species in the environment (reviewed in Kwon-Chung & Sugui 2013). *Aspergillus fumigatus* can withstand and survive in a wide range of pH and temperature and its hydrophobic cell wall allows this species to be efficiently dispersed by even slight air currents. Similarly, a number of features allow this species to be the most predominant mould species causing infections in humans (Kwon-Chung & Sugui 2013). Small conidia size allows penetration to the lower respiratory tract system and escaping clearance by mucociliary forces, presence of melanin in the cell wall enables withstanding reactive oxygen species and phagocytosis, and abundance of negatively charged sialic acid on the surface permits *A. fumigatus* to effectively bind to the basal lamina proteins once inside the host lung (reviewed in Kwon-Chung & Sugui 2013). Although conidia can be easily cleared by counteracting host mechanisms in the lung, *A. fumigatus* can cause a wide range of infections in both immunocompromised and immunocompetent individuals (Denning & Chakrabarti 2017), including an estimated annual number of 16 million pulmonary-infections (Denning *et al.* 2013, 2016) with fatal outcomes in many hundred thousand patients annually (Brown *et al.* 2012, Lowes *et al.* 2017).

Triazoles are the first-line antifungals used to treat patients suffering from aspergillosis and have brought down mortality rates to 30 % or lower in invasive aspergillosis, which is almost always fatal if untreated (Neofytos *et al.* 2009). Extensive use of azoles in various sectors ranging from agriculture and industry to clinics, however, promotes selective pressure allowing emergence of azole-resistant *A. fumigatus* (ARAF) isolates in numerous niches

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(Verweij *et al.* 2015). Subsequently, deposition of such ARAF spores in the lung of immunocompromised patients may cause azole-resistant invasive aspergillosis (IA), often in patients that have never been prescribed any azoles, resulting in treatment guidelines favouring initial treatment with liposomal amphotericin B in settings with high prevalence of azole resistance (*i.e.* > 10 %) (Thom & Church 1926). Therefore, the high mortality rate and wide range of infections together with the emergence of ARAF isolates severely complicates the management of patients suffering from aspergillosis. In this review, we discuss the current paradigm and challenges of aspergillosis, and subsequently provide suggestions to more effectively tackle these challenges utilising worldwide initiatives across multiple disciplines.

TAXONOMY AND PREVALENCE OF CLINICALLY IMPORTANT *ASPERGILLUS* SPECIES

History

The genus *Aspergillus* has a long history which dates back to Micheli's "Nova Plantarum Genera" of 1729. Micheli, being a priest, noted that the fungus he described resembled the shape of an aspergillum (sprinkler of holy water), hence the name *Aspergillus*. The genus gained more attention from 1850 onwards, because it was recognised as a causal agent of spoilage, human disease and producer of useful metabolites. Due to its economic significance, the taxonomy of the genus was studied various times in history. In 1926, Thom and Church brought all available material on *Aspergillus* together and published the first major monograph on the genus (Thom & Church 1926). This monograph was revised in 1945 (Thom & Raper 1945) and subsequently in 1965 (Raper & Fennell 1965). Their taxonomic schemes were based on macroscopic (*e.g.*, conidial colour and growth rates) and microscopic characters (vesicle shape, presence/absence of metulae). These monographs were the standard until the introduction of the molecular techniques in the 1990's. Due to DNA sequence analysis, and to a lesser extent extrolite analysis, morphologically well-defined species turn out to consist of multiple species. Nowadays, a polyphasic approach, integrating different kinds of data and information (phenotypic, genotypic and phylogenetic), is the standard for describing new species in *Aspergillus*. To date, an updated *Aspergillus* monograph is lacking; however, there are some more recent taxonomic overviews on various *Aspergillus* sections, *e.g.,* sect. *Aenei* (Varga *et al.* 2010), *Aspergillus* (Chen *et al.* 2017), *Cervini* (Chen *et al.* 2016a, 2016c), *Circumdati* (Visagie *et al.* 2014), *Clavati* (Varga *et al.* 2007), *Flavi* (Frisvad *et al.* 2019), *Flavipedes* (Hubka *et al.* 2015), *Fumigati* (Samson *et al.* 2007a), *Nidulantes* (Chen *et al.* 2016a, 2016c), *Nigri* (Samson *et al.* 2007b, Varga *et al.* 2011), *Polypaecilum* (Tanney *et al.* 2017), *Restricti* (Sklenář *et al.* 2017), *Terrei* (Samson *et al.* 2011) and *Usti* (Houbraken *et al.* 2007).

Nomenclature and *Aspergillus*

The International Code of Nomenclature for Algae, Fungi and Plants (ICN) governs the naming of fungi (McNeill *et al.* 2012). For a long time, dual nomenclature was used and asexually reproducing fungi got separate names from their sexual states. When strictly following these old rules, the name of the sexual morph had priority over the asexual morph name. For example, *Neosartorya fumigata* and *Petromyces flavus* should be used instead of the more well-known names *A. fumigatus* and *A. flavus*. The separate naming of these morphs was debated for many years and the principle "One fungus, One name" was introduced on January 1, 2013. In practice this means after that date, a fungus can only have one name. Nowadays, the name *Aspergillus* is used in a broad sense. Species producing (different) sexual morphs and previously described in teleomorph genera (*e.g.*, *Emericella, Eurotium*, *Neosartorya*, *Petromyces*) are treated as synonyms (Kocsubé *et al.* 2016). The single name nomenclature led to various name changes. Many of the clinically relevant species were already known under its current *Aspergillus* name and therefore these changes did not have a big impact in the field of medical mycology. For example, in medical mycology *Aspergillus nidulans* was already a well-known name, while *Emericella nidulans* was more commonly used in food and indoor mycology. In some cases, the species epithet already indicates the connection between the old and current name (*e.g.*, *A. chevalieri*/*Eurotium chevalieri*, *A. fischeri/Neosartorya fischeri, A. udagawae/Neosartorya udagawae*), but in other cases this is less obvious (*e.g.*, *A. glaucus*/*Eurotium herbariorum*, *A. montevidensis/Eurotium amstelodami* and *A. thermomutatus/Neosartorya pseudofischeri*). In order to help the users with these changes in nomenclature, a list of all accepted species was prepared for *Aspergillus* (and related genera) (Houbraken *et al.* 2020).

Classification of *Aspergillus*

The genus *Aspergillus* is classified in the family *Aspergillaceae*, order *Eurotiales* (Houbraken & Samson 2011). There is a long tradition of using an infrageneric classification in *Aspergillus*; these are names of taxa between the ranks of genus and species (*e.g.*, subgenera, sections, series). Using morphological characters, Raper & Fennell divided *Aspergillus* in 18 groups; however, these groups do not have any standing nomenclature and should not be used anymore (Raper & Fennell 1965). To avoid confusion and to promote taxonomic stability, a formal infrageneric classification system was introduced by Gams *et al.* (1985) and they replaced the "group" structure by a subgeneric and sectional structure. Nowadays, the genus is subdivided in six subgenera, 27 sections and 87 series (Houbraken *et al.* 2020). These formal infrageneric ranks are not commonly used in medical mycology. Articles often refer to various other informal ranks, for example "species complexes" (*e.g.*, "*A. fumigatus* species complex") (Salsé *et al.* 2019, Dos Santos *et al.* 2020), "cryptic species", "cryptic *A. fumigatus*" (Wiederhold *et al.* 2018, Rivero-Menendez *et al.* 2019a) "species clades" (*e.g.*, *A. fumigatus*-clade) (Balajee *et al.* 2005, 2007a), "*sensu lato*" (*e.g.*, *A. fumigatus sensu lato*) (Li *et al.* 2014, Hagiwara *et al.* 2019) and "*sensu stricto*" (*e.g.*, *A. fumigatus sensu stricto*) (Li *et al.* 2014, Monteiro *et al.* 2019)). The main disadvantage of using these informal ranks is lack of consensus. For example, it is not clear whether "*A. fumigatus sensu lato*", "*A. fumigatus* species complex", "cryptic *A. fumigatus*" and the "*A. fumigatus*-clade" are actually representing the same (group of) species. It is therefore recommended to use, when possible, a formal classification system of subgenera, sections and series. In the case of *A. fumigatus*, it is recommended to refer to *A. fumigatus* (the

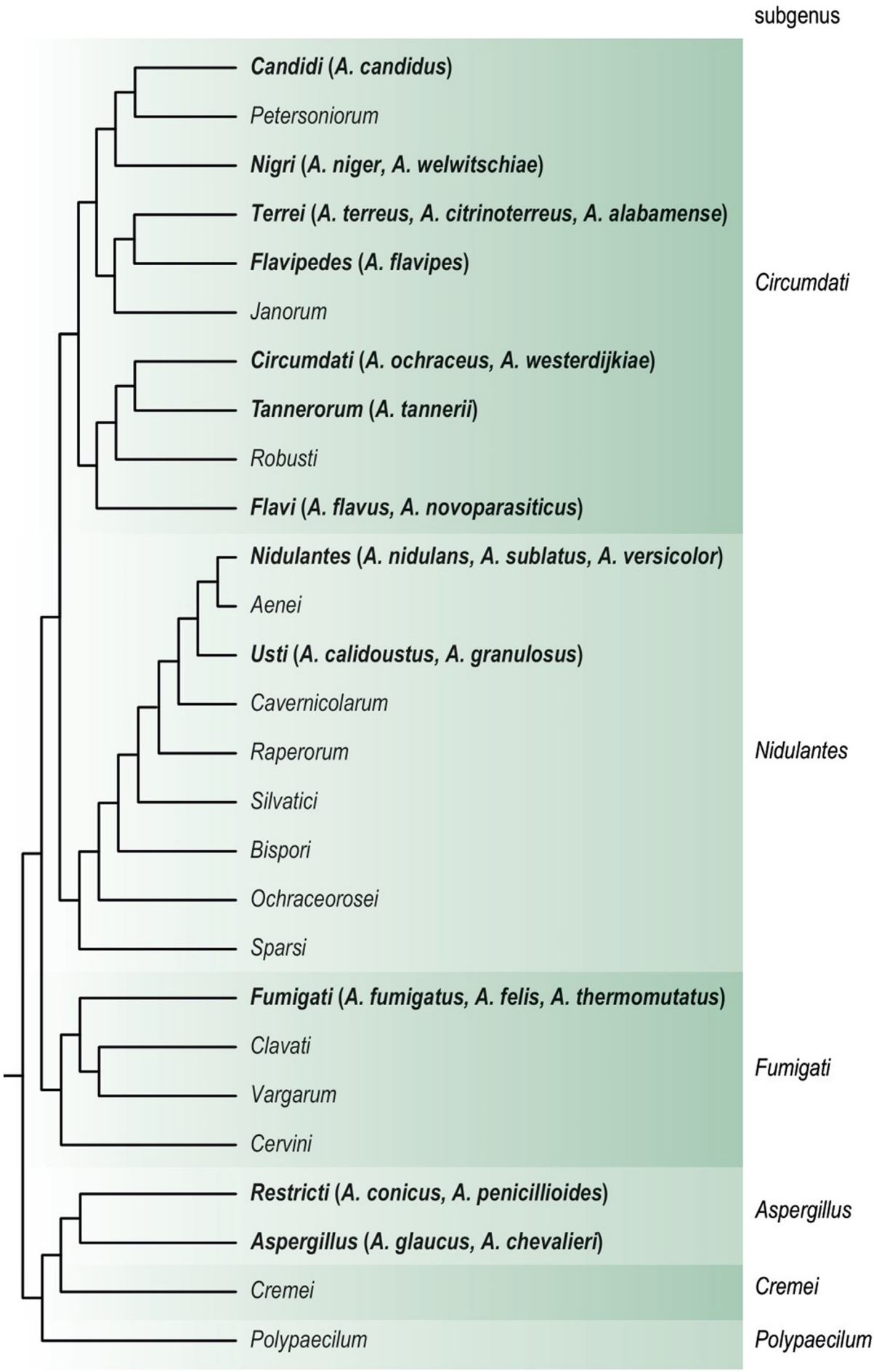


Fig. 1. Cladogram of the genus *Aspergillus* and the relationship between sections and subgenera. A selection of the species mentioned in the text are given in brackets in bold font after the section name. Adopted from Houbraken *et al.* (2020) with permission.

species), series *Fumigati* (for *A. fumigatus* and the related species *Aspergillus fischeri*, *A. fumigatiaffinis*, *A. fumigatus*, *A. fumisynnematus*, *A. laciniosus*, *A. lentulus*, *A. novofumigatus*, *A. oerlinghausensis*, *A. spinosus*, *A. takakii*) or section *Fumigati* (59 species) (Houbraken *et al.* 2020).

Identification of *Aspergillus* species from pure culture

In the last decade, there is steep increase of the number of accepted *Aspergillus* species (Houbraken *et al.* 2020). The driving forces behind this steep increase are twofold: firstly, there is a large diversity and high interest in this genus and secondly, phenotypically well-known species are turn out to be species complexes that are genetically and evolutionary distinct (Chen *et al.* 2016a, 2016c, 2017, Houbraken *et al.* 2016a, Sklenář *et al.* 2017). Morphology was for a long time the mainstay in *Aspergillus* identification (Raper & Fennell 1965). As morphologically well-defined species turned out to be species complexes, accurate phenotype-based identification became more difficult and unreliable. There are often only small differences between species within a complex and sometimes they need to be grown on special agar media to observe those differences. Identification based on phenotypic characters is therefore challenging, even for experienced mycologists and (well-trained) staff of routine labs. These phenotypically similar species are also referred to as "cryptic species"; however, they can be identified using a molecular based approach. These phenotypically closely related (cryptic) species can have strikingly different patterns of antifungal susceptibility patterns against the most important antifungals, including triazoles and amphotericin B (AMB), and some of these species are intrinsically resistant or have acquired resistance against these antifungals (Alastruey-Izquierdo *et al.* 2013, 2014, Escribano *et al.* 2013, Negri *et al.* 2014, Iatta *et al.* 2016, Heo *et al.* 2017, Talbot & Barrs 2018, Zoran *et al.* 2018, Salah *et al.* 2019, Mendoza *et al.* 2020, Glampedakis *et al.* 2021). For correct identification of *Aspergillus* species, calmodulin gene sequencing is recommended (Samson *et al.* 2019), and partial β-tubulin gene sequencing can be used as an alternative. ITS sequencing lacks resolution and is therefore not suitable. The public databases are well-stocked with calmodulin gene sequences and 96.9 % of the 446 accepted *Aspergillus* taxa are represented with a calmodulin gene sequence in GenBank (Houbraken *et al.* 2020). Actually, the taxonomic position of the species lacking a calmodulin sequence needs to be determined (Houbraken *et al.* 2020) and are unlikely to be relevant in medical mycology. Besides an overview of the accepted species, also calmodulin and β-tubulin references sequences are given.

Since its introduction into clinical microbiology diagnostics, MALDI-TOF MS has become the standard workhorse system for the identification of bacteria and yeasts (Kostrzewa 2018). Because of their rigid cell wall and the phenotypic variability by sporulation, the identification of moulds has been shown to be more challenging. Different approaches for sample preparation have been developed to overcome these hurdles, *e.g.,* tube extraction of proteins before spotting on the MALDI-TOF MS target or liquid culturing to avoid sporulation. A prerequisite of successful identification of moulds are extensive libraries containing high-quality reference spectra of well characterised strains. This has led to a number of dedicated, user-specific databases and database supplements besides the libraries supplied by manufacturers (Sanguinetti & Posteraro 2017, Patel 2019). A study of US academic centres using a database established at the NIH, a solid media extraction method and a challenge set of 80 clinical mould isolates demonstrated the requirement of instrument optimisation and high standardisation for mould identification across different laboratories (Lau *et al.* 2019). MALDI-TOF MS has been successfully applied to *Aspergillus* spp. identification in several studies. Thereby, it could be demonstrated that many but not all rare and cryptic species can be correctly identified if they are represented well in the according database (Vidal-Acuña *et al.* 2018, Imbert *et al.* 2019, Américo *et al.* 2020). Closely related *Aspergillus* species are sometimes difficult to differentiate by MALDI-TOF MS because of their similar spectral pattern, today, but this can be improved by further extension of databases and utilisation of alternative identification algorithms.

Taxonomic notes on *A. fumigatus* and other clinically relevant Aspergilli

Aspergillus section Fumigati

The species in this section produce uniseriate, columnar conidial heads in shades of green and flask shaped vesicles (Raper & Fennell 1965). Traditionally, the identification of these related species is performed using the colony patterns and the morphology of the conidiogenous structures, conidia, ascomata and ascspores (Raper & Fennell 1965). However, clinical isolates can be markedly abnormal being more floccose with fewer conidia and more recent taxonomic studies showed that section *Fumigati* species can have a highly similar morphology. For accurate identification, a sequence-based approach is therefore recommended (Samson *et al.* 2007a). *Aspergillus* section *Fumigati* includes 63 species and the species of the section are thermotolerant. *Aspergillus fischeri, A. fumigatus* and *A. oerlinghausenensis* are able to grow at 50 °C (Houbraken *et al.* 2016b), while other species of the section have lower maximum growth temperatures (*e.g.,* 45 °C: *A. fumigatiaffinis*, *A. fumisynnematus*, *A. lentulus*, *A. novofumigatus*; 42 °C: *A. felis*) (Balajee *et al.* 2007a). The sexual morph is of the neosartorya-type (Samson *et al.* 2007a). The most well-known member of this section is *A. fumigatus*, though other species in the section are also clinically relevant: *A. felis*, *A. fischeri, A. fumigatiaffinis*, *A. fumisynnematus*, *A. hiratsukae*, *A. laciniosus*, *A. lentulus*, *A. novofumigatus*, *A. pseudoviridinutans*, *A. spinosus*, *A. thermomutatus*, *A. udagawae* and *A. viridinutans* (Sugui *et al.* 2014, Frisvad & Larsen 2016). Taxonomic evaluation of *A. pseudofelis* and *A. parafelis* (also known as *A. felis*) showed that these species are included in the genetically diverse *A. felis* (Hubka *et al.* 2018) (Fig. 1).

Aspergillus fumigatus is reported as the most prevalent species from this section in different countries (Binder & Lass-Flörl 2013). Other species of section *Fumigati*, the so-called cryptic *A. fumigatus*, have been increasingly identified in the clinical setting in the last two decades, which is because of the increasing use of the polyphasic approach for the discrimination and identification of *Aspergillus* species in clinical samples. Based on multilocus comparative sequence analysis, other species from this section are recovered from 3 to 6 % of patients with IA. Among cryptic species, *A. felis*, *A. lentulus*, *A. thermomutatus* and series *Viridinutantes* members (= *A. viridinutans*

complex) are the most common isolates from clinical disease in human (Balajee *et al.* 2009, Alastruey-Izquierdo *et al.* 2013, 2014, Escribano *et al.* 2013, Negri *et al.* 2014, Sabino *et al.* 2014, Frisvad & Larsen 2016, Talbot *et al.* 2018, Paccoud *et al.* 2019, Yagi *et al.* 2019).

Aspergillus fumigatus biosynthesizes a variety of secondary metabolites such as fumagillin, fumitoxins, fumigaclavines A & C, fumitremorgins, gliotoxin, trypacidin, pseurotins, helvolic acid, pyripyropens, methyl-sulochrin, verruculogen, fumiquinazolines. Several of these metabolites may cause serious health hazard (Frisvad & Samson 1990, Fujimoto *et al.* 1993, Larsen *et al.* 2007, Frisvad & Larsen 2016), though none of them are actually regulated mycotoxins. Some of these metabolites are involved in impairing the host immune system (Steenwyk *et al.* 2020) *e.g.,* gliotoxin has been shown to inhibit the host immune response (Sugui *et al.* 2007, Spikes *et al.* 2008).

Other Aspergillus sections with clinically relevant species

Besides the section *Fumigati* species, a wide variety of other Aspergilli are clinically relevant. Most of them belong to the species-rich sections *Flavi*, *Nidulantes*, *Nigri*, *Terrei* and *Usti*. The taxonomy of these sections is well-studied and correct identification using sequence data should therefore not be problematic (Houbraken *et al.* 2020).

The taxonomy of section *Flavi* was recently updated and contains 35 species (Visagie *et al.* 2014, Frisvad *et al.* 2019, Houbraken *et al.* 2020), of which nine species are known to cause infection in humans (Hedayati *et al.* 2007, Frisvad *et al.* 2019, Rudramurthy *et al.* 2019, Alshehri & Palanisamy 2020). *Aspergillus flavus* is the main and most commonly occurring species of the section. Most of the reports of *A. oryzae* in clinical settings are likely to be erroneous. *Aspergillus oryzae* is the domesticated form of *A. flavus* and they can be regarded as conspecific. It is impossible to reliably differentiate *A. oryzae* and *A. flavus* using morphology and calmodulin or β-tubulin gene sequencing. The differentiation between both species is mainly driven by an applied aspect: *A. oryzae* is extensively used in food fermentations (*e.g.,* soy sauce, sake) and for the production of enzymes, and these industries do not want to use the name *A. flavus*, which has a strong association with aflatoxin production. As a consensus, strains that do not produce aflatoxin and have a food fermentation or biotechnological background can be identified as *A. oryzae*; wild-type strains are *A. flavus*.

The clinically most important species of section *Terrei* is *A. terreus*, which is the second or third most common cause of IA in immunocompromised patients (Lass-Flörl *et al.* 2005, Blum *et al.* 2008). Of the 17 accepted species in section *Terrei* (Houbraken *et al.* 2020), five are human pathogens (*A. alabamensis*, *A. citrinoterreus*, *A. floccosus*, *A. hortae* (= *A. hortai*) and *A. neoafricanus*) (Zoran *et al.* 2018, Lackner *et al.* 2019). According to a recent report, infections caused by the *A. terreus* species complex were identified in 21 countries and 38 centres, and account for 5.2 % of all mould infections (Risslegger *et al.* 2017). However, a high incidence of *A. terreus* infections was reported in Innsbruck (Austria) and Houston (USA) (Lass-Flörl *et al.* 2007).

Species belonging to section *Nigri* ("the black Aspergilli") are phenotypically very similar. Eight (*A. brasiliensis*, *A. carbonarius*, *A. japonicus*, *A. luchuensis* (= *A. acidus*), *A. niger* (= *A. foetidus*), *A. tubingensis*, *A. uvarum*, *A. welwitschiae* (= *A. awamori*) of the 30 accepted species in section *Nigri* are reported to cause infections in humans. The identified pathogenic species from this section are generally reported as the third leading causative agents of IA (Samson *et al.* 2014, Huang *et al.* 2017). Within this section, *A. niger sensu stricto* is the most prevalent clinical isolate (68.4 % cases *vs. A. tubingensis*, 31.6 % cases) (Balajee *et al.* 2009). However, recent analyses based on β-tubulin and calmodulin gene sequencing revealed a shift toward other cryptic species, including *A. tubingensis*, and *A. welwitschiae*, in different countries (Iatta *et al.* 2016, Hedayati *et al.* 2019, Alshehri & Palanisamy 2020, Carrara *et al.* 2020, Takeda *et al.* 2020).

The majority of the 74 accepted species in section *Nidulantes* are isolated from the soil, plant material, or the indoor environment (Sklenář *et al.* 2020). Eleven species have been isolated from patients with *Aspergillus* infections, of which *A. nidulans* was reported as the main agent of IA in different countries (Gabrielli *et al.* 2014, Chrenkova *et al.* 2018, Seyedmousavi *et al.* 2018, Tavakoli *et al.* 2020). *Aspergillus nidulans* was also reported as the second most frequently encountered mould in patients with chronic granulomatous disease characterised by sudden invasive features (Blumental *et al.* 2011, King *et al.* 2016b, Khalid & Ali 2018). Section *Versicolores* is a synonym of section *Nidulantes*, and series *Versicolores* is nowadays used. The clinically relevant species *A. sydowii* and *A. creber* belong to series *Versicolores* (Borgohain *et al.* 2019, Alshehri & Palanisamy 2020); of all 25 members in the section *Usti*, *A. calidoustus* is most often reported as the causal agent of invasive infections. Prior to the description of *A. calidoustus*, clinical strains were attributed to *A. ustus* and *A. calidoustus* are easy to differentiate, since the latter grows rapidly at 37 °C, while the former does not (Balajee *et al.* 2007a, Varga *et al.* 2008). Other members of the section isolated from proven/probable IA cases include *A. granulosus*, *A. pseudodeflectus* and *A. ustus*; *A. insuetus*, *A. keveii*, *A. puniceus*, *A. pseudodeflectus* and *A. ustus* were reported from respiratory samples (Fakih *et al.* 1995, Glampedakis *et al.* 2021).

In addition to the species mentioned above, other taxa can also cause infections in humans. Others include *A. chevalieri*, *A. costiformis*, *A. glaucus*, *A. montevidensis*, *A. proliferans* and *A. pseudoglaucus* (sect. *Aspergillus*) (Aznar *et al.* 1989, Naidu & Singh 1994, Traboulsi *et al.* 2007, Hubka *et al.* 2012, Alshehri & Palanisamy 2020); *A. insulicola*, *A. melleus*, *A. ochraceopetaliformis*, *A. ochraceus*, *A. persii*, *A. sclerotiorum*, *A. subramanianii* and *A. westerdijkiae* (sect. *Circumdati*) (Novey & Wells 1978, García-Martos *et al.* 2001, Harima *et al.* 2004, Brasch *et al.* 2009, Zotti *et al.* 2010, 2015, Hubka *et al.* 2012, Babamahmoodi *et al.* 2015, Bongomin *et al.* 2018, Seyedmousavi *et al.* 2018, Amri *et al.* 2020); *A. flavipes* (sect. *Flavipides*) (Seyedmousavi *et al.* 2018); *A. tanneri* (sect. *Tanneroru*m) (Seyedmousavi *et al.* 2018); *A. candidus* (sect. *Candidi*) (Bongomin *et al.* 2018); and *A. penicillioides* and *A. conicus* (sect. *Restricti*) (Sklenář *et al.* 2017).

VIRULENCE, IMMUNOLOGY AND PATHOGENESIS OF *ASPERGILLUS*

In its natural environment, *Aspergillus* behaves as a saprobe that survives under different stress conditions. Likewise, during human infection, it has evolved adaptive mechanisms that allow it to withstand the unfavourable conditions in the lungs and to

counter environmental changes in temperature, pH, water and nutrient balance, oxidative stress, and host molecules with antifungal properties.

Among the many virulence traits exhibited by *Aspergillus*, its plasticity in nutrient acquisition and metabolism confers a major advantage for growth during fungal infection under conditions of limited nutrient availability (Brock 2009, Blatzer & Latgé 2017). In addition, in experimental models of *A. fumigatus* infection, sites of hypoxia are commonly observed in the lungs, highlighting a remarkable ability to survive and thrive in conditions of low oxygen (Grahl *et al.* 2011, Kowalski *et al.* 2019). Besides these and several other relevant traits elicited in the context of infection (reviewed in Latgé & Chamilos 2019), the cell wall is a unique virulence factor, since it protects *A. fumigatus* from external aggression, while at the same time, it plays an active role in infection by influencing and modulating the immune response of the host (Latgé *et al.* 2017, van de Veerdonk *et al.* 2017). Owing to its dynamic structural properties according to morphotype, growth stage, and environmental conditions, the fungal cell wall is the main source of fungal ligands that activate the immune system (Latgé 2010). The physical barrier of the respiratory tract affords the first line of defence against inhaled conidia of *Aspergillus*, after which the respiratory epithelium is the initial point of contact with inhaled conidia (Filler & Sheppard 2006). Indeed, an increasing body of evidence has revealed a critical role of the airway's epithelium in fungal clearance (Amich *et al.* 2020, Seidel *et al.* 2020) and production of cytokines and antimicrobial peptides (Bellanger *et al.* 2009, Sharon *et al.* 2011, Richard *et al.* 2018). Under certain conditions conidia escape the respiratory epithelium and are then challenged by cells of the innate immune system, including resident alveolar macrophages and dendritic cells (van de Veerdonk *et al.* 2017). Germinating conidia that escape macrophages are eliminated by recruited neutrophils and monocytes. Neutrophil extracellular traps (NETs) contribute to the innate host defence *in vivo* and neutrophils exert a considerable variety of antifungal effector functions, which include recognition, phagocytosis, intracellular clearance mediated by both oxidative and non-oxidative mechanisms, secretion of antimicrobial molecules and the release of neutrophil extracellular traps (NETs) (Urban & Backman 2020). Failure to prevent conidial germination results in hyphal growth, tissue invasion, and marks the initiation of fungal disease. Innate immune cells express a vast repertoire of pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns in the fungus and activate effector functions, including phagocytosis and the production of proinflammatory cytokines and chemokines that orchestrate innate and adaptive immune responses (Patin *et al.* 2019). IL-8, also known as neutrophil chemotactic factor, is produced by macrophages and epithelial cells as an important chemoattractant for neutrophils, also during early phases of IA, where conidia are killed by local alveolar macrophages, and has been extensively used as biomarker for invasive aspergillosis (Winn *et al.* 2003, Camargo & Husain 2014, Gonçalves *et al.* 2017, Heldt *et al.* 2017, 2018, Jenks *et al.* 2019d). Up-regulation of gene transcription by *A. fumigatus* proteases has been suggested as cause of increased release of IL-8 by A549 pulmonary epithelial cells and primary epithelial cells (Borger *et al.* 1999). Other studies have shown that *in vitro* opsonization of *A. fumigatus* conidia with H-ficolin, L-ficolin and M-ficolin, which play essential roles in pathogen recognition and complement activation through the lectin pathway, potentiate IL-8 secretion of A549 lung epithelial cells (Houser *et al.* 2013, Bidula *et al.* 2015, Ghufran *et al.* 2017).

The family of C-type lectin receptors (CLRs) is the best-studied with regard to antifungal immunity (Brown *et al.* 2018). For example, the importance of dectin-1 in the recognition of β-1,3-glucan and activation of downstream immune responses has been confirmed in patients with recurrent fungal infections carrying an early stop codon polymorphism (Ferwerda *et al.* 2009). This polymorphism results in a truncated form of dectin-1 lacking several amino acids, with a detrimental effect on recognition of β-1,3-glucan and cytokine production after fungal stimulation (Ferwerda *et al.* 2009, Cunha *et al.* 2010). As a result, this polymorphism was found to predispose hematopoietic stem-cell transplant (HSCT) recipients to the development of IA in different patient cohorts (Cunha *et al.* 2010, Chai *et al.* 2011, Fisher *et al.* 2017). More recently, another CLR named MelLec was identified as the receptor for fungal melanin (Stappers *et al.* 2018). Macrophages from carriers of a polymorphism in the cytoplasmic tail of MelLec displayed a generalised defect in the production of cytokines after fungal stimulation. Likely owing to this defect, HSCT recipients receiving grafts from affected donors displayed a markedly increased risk for invasive pulmonary aspergillosis (IPA) after transplantation (Bassetti *et al.* 2020, Donnelly *et al.* 2020).

The efficiency of fungal recognition also relies largely on the opsonization by different soluble pattern recognition molecules, including collectins, pentraxins, ficolins and components of the complement pathway (Bidula & Schelenz 2016). One molecule that has received a great deal of recent attention in the field of aspergillosis is the long pentraxin-3 (PTX3) (Foo *et al.* 2015). This molecule binds microbial moieties from a wide range of microorganisms, including *A. fumigatus* (Garlanda *et al.* 2002). Accordingly, genetic variation in PTX3 was identified as a major risk factor for IPA after HSCT (Cunha *et al.* 2014), an association that was validated in independent cohorts of recipients of HSCT (Fisher *et al.* 2017) and solid organ transplant (Cunha *et al.* 2015, Wojtowicz *et al.* 2015), as well as in patients with chronic obstructive pulmonary disease (Cunha & Carvalho 2018, He *et al.* 2018). Mechanistically, genetic variants in PTX3 compromised the normal expression of the protein in the lungs and, at a cellular level, the antifungal effector mechanisms of neutrophils (Cunha *et al.* 2014). The impact of PTX3 deficiency on neutrophil function was confirmed in a recent study describing a similar association in patients with acute myeloid leukemia undergoing chemotherapy courses without pre-existing neutropenia (White *et al.* 2018).

The interaction of *Aspergillus* with the immune system is being harnessed to propose novel and improved fungal diagnostics, but also the implementation of clinical models aimed at the effective prediction of infection in high-risk patients. A recent study evaluating the combination of multiple genetic and clinical factors into a predictive model has demonstrated that such information could be used to successfully guide pre-emptive therapy in haematological patients (White *et al.* 2018). Besides improved diagnostics, functional analyses of genetic variation influencing susceptibility to aspergillosis may assist in the design of innovative and personalised immunotherapeutic approaches. This is illustrated by the preclinical evidence showing that genetic PTX3 deficiency can be rescued by the administration of the recombinant protein, a finding that supports its personalised use in specific patients at high-risk of infection. In conclusion, the success of novel diagnostic and

immunotherapeutic approaches for aspergillosis may benefit from personalisation based on the interindividual variability in antifungal immune function.

CLINICAL SIGNIFICANCE OF *ASPERGILLUS*

Infection in humans

Aspergillus is the most common cause of mould infections in humans and can cause a variety of serious diseases in both immunocompetent and immunocompromised patients (Lass-Flörl 2019). The most clinically relevant *Aspergillus* species is *A. fumigatus*, followed by *A. flavus*, *A. terreus* and *A. niger*. Non-invasive infections in immunocompetent patients (*e.g.* with cystic fibrosis or post-tuberculosis) are allergic sinusitis or allergic bronchopulmonary aspergillosis (ABPA), fungal balls in the sinus or lung, chronic pulmonary aspergillosis, otitis externa or onychomycosis (Denning *et al.* 2018). For invasive infections, the respiratory tract is the most common primary site of IA –due to inhalation of conidia– but any organ might be involved as a single organ infection or as part of dissemination. Sino-nasal and cerebral aspergillosis may occur particularly in immunocompromised patients (Reischies & Hoenigl 2014). *Aspergillus* endocarditis is rare and risk factors include prior valve replacement, indwelling central venous catheters or broad-spectrum antibiotic treatment (Aldosari *et al.* 2020). Hematogenous spread to the spleen leads to either infarction or abscesses (Smolovic *et al.* 2018). Aspergillosis of the kidney is rare and derives from hematogenous dissemination or ascending from pan-urothelial aspergillosis or secondary to obstructive uropathy. Gastrointestinal tract IA may occur when the mucosal barrier is impaired (Chasan *et al.* 2013). Other rare manifestations of IA are (vertebral) osteomyelitis, arthritis, or subacute thyroiditis. Endophthalmitis may be consequent to intraocular surgery or trauma of the eye or after hematogenous spread and is associated with poor ocular prognosis (Dave *et al.* 2020).

Cutaneous aspergillosis may be caused by inoculation into disrupted skin or secondary to hematogenous dissemination (Lass-Flörl 2019). Primary extrapulmonary invasive aspergillosis often requires surgery in addition to systemic antifungal therapy (Reischies & Hoenigl 2014, Dave *et al.* 2020). Depending on the type of immunosuppression of the host, invasive pulmonary aspergillosis may present primarily angio-invasive in those with neutropenia, or primarily airway-invasive in those with corticosteroid associated immunosuppression, resulting in distinct radiological and clinical manifestations (Bergeron *et al.* 2012, Jenks *et al.* 2019b). The most common site of hematogenic spread is the central nervous system leading to brain abscess, stroke or less frequent to meningitis, and associated with devastating mortality rates (Hoenigl & Krause 2013).

Emergent susceptible population to acquire pulmonary aspergillosis

Mould-active prophylaxis has shown some success in reducing IA in patients with traditional risk factors for IA, such as those with underlying hematologic malignancy and prolonged neutropenia (Cornillet *et al.* 2006, Duarte *et al.* 2014). However, the prevalence of IA continues to increase in non-neutropenic patients with severe underlying diseases, including those in intensive care units (Pappas *et al.* 2010, Eigl *et al.* 2015, Bassetti *et al.* 2018, Schauwvlieghe *et al.* 2018b), solid organ transplant recipients (Lewis & Kontoyiannis 2009), those receiving systemic glucocorticoids (Chamilos *et al.* 2018), those with underlying structural lung damage (Prattes *et al.* 2014), those with advanced liver cirrhosis and liver failure (Prattes *et al.* 2017), those receiving tyrosine kinase inhibitors such as ibrutinib (Lenczuk *et al.* 2018), those with solid cancers (Yan *et al.* 2009, Bassetti *et al.* 2018), and others (Guinea *et al.* 2010, Prattes *et al.* 2014, Bassetti *et al.* 2018, Ghez *et al.* 2018).

Aspergillus species, especially *A. fumigatus*, can cause co-infection with viruses, including cytomegalovirus, and –importantly– influenza virus leading to complication of management of patients inflicted (Schauwvlieghe *et al.* 2018b). In one multicentre study from the Netherlands and Belgium, invasive pulmonary aspergillosis was diagnosed in 83 (19 %) of 432 patients admitted with influenza to the ICU, a median of 3 days after admission to the ICU (Schauwvlieghe *et al.* 2018b), and independently associated with mortality. In another study from Canada, IA complicated only 7.2 % of influenza associated respiratory failure ICU admissions, although the rate varied between 0 and 23 % between influenza seasons (Schwartz *et al.* 2020).

In November 2019, a novel virus termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first identified as the causative agent of pneumonia from a cluster of individuals with pneumonia in Wuhan City, Hubei province, China (WHO 2020). Since the first reported case, SARS-CoV-2, which causes the disease now called coronavirus disease 2019 (COVID-19), has spread throughout China and to almost every country in the world as of May 2020. Although the vast majority of COVID-19 cases are mild to moderate, up to 20 % of patients with symptomatic COVID-19 may develop acute respiratory distress syndrome (ARDS) (Wang *et al.* 2020, Wu & McGoogan 2020, Xu *et al.* 2020). There is increasing evidence that COVID-19 patients are at risk of developing COVID-19 associated invasive pulmonary aspergillosis (CAPA) co-infection, with more than 100 cases reported to date (Blaize *et al.* 2020, Dupont *et al.* 2020, Gangneux *et al.* 2020, Hoenigl 2021, Koehler *et al.* 2020b, Lescure *et al.* 2020, Mitaka *et al.* 2020, van Arkel *et al.* 2020, Verweij *et al.* 2020, Bartoletti *et al.* 2021, Prattes *et al.* 2021, White *et al.* 2021). The pathophysiology of COVID-19 is not well understood, but leukopenia, lymphopenia and T-cell perturbations, including immune dysregulation impacting Th2 as well as Th1 responses in severe COVID19, are frequently observed among symptomatic patients (Chen *et al.* 2020a, 2020b, Huang *et al.* 2020, Zheng *et al.* 2020), and may predispose patients to fungal diseases. Furthermore, the utilisation of – often high dose – systemic and inhaled glucocorticoids, which may further predispose to opportunistic infections such as CAPA, has been described in close to 50 % of patients with COVID-19-associated ARDS (Wang *et al.* 2020). This immune dysregulation together with epithelial lung damage stemming from COVID-19 immunopathology may facilitate *Aspergillus* superinfection.

From Wuhan, epidemiological studies indicate that invasive fungal infections may occur in 4–5 % of COVID-19 episodes requiring ICU admission (Baxter *et al.* 2011, Yang *et al.* 2020). In a cohort from China, fungal infections were diagnosed in seven (3 %) out of 221 COVID-19 patients, all of whom were admitted to the ICU (Zhang *et al.* 2020). Given that in Wuhan,

galactomannan (GM) testing is rarely available and fungal diagnostics are sparse (Chindamporn *et al.* 2018), this is likely an underestimate of the true burden of IA in patients with SARS-CoV-2 requiring ICU admission. A study from the United Kingdom reported a 14.1 % incidence of pulmonary aspergillosis among 135 ICU patients (White *et al.* 2021). Higher rates of CAPA were recently reported in a single centre study from the Netherlands, where a high incidence (19.4 %) of putative aspergillosis was observed in a cohort of 31 mechanically ventilated ICU patients with COVID-19 (van Arkel *et al.* 2020). In another study from Germany, five of 19 (26 %) consecutive critically ill COVID-19 patients with ARDS were diagnosed with putative CAPA, highlighting that rates may be comparable to those observed in association with severe influenza (Koehler *et al.* 2020b). In a study from Italy, probable CAPA was diagnosed in 30 (27.7 %) patients after a median of 4 (2–8) days from intensive care unit (ICU) admission (Bartoletti *et al.* 2021). In a study from France, putative CAPA was reported in 33 % of 27 critically ill COVID-19 patients in an enriched population undergoing bronchoscopy (Alanio *et al.* 2020). Finally, the highest rate of CAPA has been noted in Belgium reaching 35 % (7/20) of the COVID-19 patients presented with ARDS (Rutsaert *et al.* 2020). The vast majority of reported CAPA cases lacked EORTC/MSGERC host factors, highlighting the need for improved criteria for defining IPA in non-neutropenic patients, as reported elsewhere (Jenks *et al.* 2019b). Additional cases of fatal CAPA were reported from Argentina, Australia, Austria, Brazil, France, Ireland, Italy, Pakistan, Switzerland, United States and many other countries (Blaize *et al.* 2020, Mitaka *et al.* 2020, Prattes *et al.* 2021). Importantly, three cases of azole resistance have been reported, indicating that ARAF is emerging also among the ICU population at risk for IPA (Meijer *et al.* 2020, Ghelfenstein-Ferreira *et al.* 2021, Mohamed *et al.* 2021).

As clinical presentation and imaging findings of COVID-19 and IPA may overlap (fever, shortness of breath, cough, unspecific infiltrates and consolidations, halo sign), biomarker and culture based diagnostic work-up is essential. Serum GM may have imperfect sensitivity of 20 % and below (Alanio *et al.* 2020, Koehler *et al.* 2020b). While reasons for the lower sensitivity in CAPA versus influenza associated pulmonary aspergillosis are unknown, treatment with chloroquine, which exhibits *in vitro* activity against *A. fumigatus* (Henriet *et al.* 2013), may have explained the lower sensitivity in some of the earlier studies, given that exposure to antifungals is a well-known factor that decreases the sensitivity of GM-testing and may explain the lower sensitivity compared to influenza associated IPA (Eigl *et al.* 2015).

Future studies are needed to evaluate other blood tests for CAPA, including *Aspergillus* PCR (Egger *et al.* 2020), β-D-glucan (Heldt *et al.* 2018), and the two newly CE-marked point of care tests, the *Aspergillus* GM lateral flow assay (LFA) and the *Aspergillus*-specific lateral flow device test (Eigl *et al.* 2015, Jenks *et al.* 2019c, 2019e, Mercier *et al.* 2020, Wahidi *et al.* 2020). Further complicating diagnosis of CAPA is the extremely limited role of bronchoscopy in COVID-19 as this aerosol generating procedure increases the risk of exposure for patients and personnel (Jenks *et al.* 2020), although detailed instructions on how to safely perform bronchoscopy have been published (Koehler *et al.* 2020c). In some centres, however, collection of tracheal aspirates remains the preferred method for diagnosis. Although *Aspergillus* can be detected in sputum and tracheal aspirates in CAPA-patients, its presence might reflect oral pharyngeal colonisation as *Aspergillus* is considered a core component of the basal oral mycobiome (Krüger *et al.* 2019). Importantly, GM-testing, presented in detail below, which is an important tool for IPA diagnosis in BALF specimens and represents active growth (Eigl *et al.* 2017), is not validated for upper respiratory tract specimens.

These early findings suggest IA may be an important and underrecognised complication of SARS-CoV-2 infection, due to the absence of typical host factors. Since bacterial and fungal superinfections are difficult to distinguish from severe COVID-19 based on clinical or imaging findings, histopathology has a central role in determining prevalence and also outcomes of CAPA (reviewed in Arastehfar *et al.* 2020a). However, autopsies of COVID-19 patients are rarely performed due to the risk of aerosol transmission. Criteria for defining COVID-19 associated aspergillosis have been developed and will help classifying this important disease (Koehler *et al.* 2020a). The frequency of post-COVID-19 aspergillosis is likely to differ significantly between hospitals and geographic sites, and environmental factors may also play a large role in increasing exposure beyond what would normally be encountered within hospitals and ICUs. The rapid spread of COVID-19 to a non-immune population has been seen in temporary facilities/hospitals rapidly assembled that do not adhere to the rigorous ventilation requirements that are present within permanent hospitals. These temporary sites are essential to increase healthcare capacity; however, dust and construction-related increases in ambient air spore counts will very likely increase patient colonisation with *Aspergillus* and other fungal species predisposing to infection. Finally, drug-drug interactions may limit the use of voriconazole (Jenks *et al.* 2019a), the gold-standard treatment for IA in the ICU. Future studies need to evaluate effectiveness of isavuconazole (Jenks *et al.* 2018), and new antifungals currently under development like fosmanogepix or olorofim (Kupferschmidt 2019), which may have comparable efficacy without the same burden of drug-drug interactions, while other drugs such as rezafungin or ibrexafungerp may offer options for combination therapy or even prophylaxis.

DIAGNOSIS OF ASPERGILLOSIS IN CLINIC – SEROLOGY TO PCR

For the diagnosis of IA, culture and microscopy are essential, but show limited sensitivity. Detection of the fungal cell wall component galactomannan (GM) has therefore become the imperfect gold standard (Hoenigl *et al.* 2012, 2013b, Duettmann *et al.* 2014, Eigl *et al.* 2017, Rawlings *et al.* 2019) as it is more sensitive than culture. Galactomannan is a polysaccharide that exists primarily in the cell walls of *Aspergillus* species (Zhou *et al.* 2017) and a commercially-available double sandwich enzyme immunoassay (EIA) utilises the monoclonal antibody EB-A2 (Platelia™, Bio-Rad, Marnes-la-Coquette, France) to detect the GM antigen. It is approved by the U.S. Food and Drug Administration (FDA) for testing of GM from serum and bronchoalveolar fluid (BALF). This antibody detects multiple epitopes on galactofuranose side chains that link to the mannan backbone (Kudoh *et al.* 2015), although galactofuranose is not specific to *Aspergillus* and is present in other fungi such as *Fusarium* species (Tortorano *et al.* 2012), *Penicillium* species (Huang *et al.* 2007), and *Histoplasma* species (Wheat *et al.* 2007). The current sensitivity and specificity of BALF GM is 82 % and 81 %,

respectively (Rawlings *et al.* 2019). The optimal optical density index (ODI) threshold is debatable, although the FDA considers an ODI of $\geq$ 0.5 to be positive for GM in both serum and BALF.

Galactomannan from BALF has shown better diagnostic performance for IA than GM from blood, particularly in patients on mould-active antifungal prophylaxis (Heldt *et al.* 2018). In a systematic review investigating the accuracy of GM from BALF for the diagnosis of IA in immunocompromised patients, the sensitivity and specificity of GM was 0.88 and 0.81, respectively, at an ODI of 0.5; at an ODI of 1.0 the sensitivity was 0.78 and specificity 0.93 (de Heer *et al.* 2019). Particularly in non-neutropenic patients a higher cut-off of 1.0 ODI in BALF may be preferable (Prattes *et al.* 2014) as false-positive results may occur with the lower cut-off (Martinelli *et al.* 2019). Given the airway-invasive growth, BALF GM is always the preferable option for IA diagnosis in non-neutropenic patients, and GM from BALF has found to be superior to GM from serum (Bassetti *et al.* 2020).

Molecular tests such as PCR (Heldt *et al.* 2018, Prattes *et al.* 2018, Jenks *et al.* 2019f, Rawlings *et al.* 2019) have emerged as alternative options to diagnose IA and are widely used (Hoenigl *et al.* 2014b, Buchheidt *et al.* 2017), although there is a lack of standardisation (White *et al.* 2010) and a large variation in diagnostic performance across studies and settings (White *et al.* 2015b, Springer *et al.* 2016). PCR is most useful in high-risk groups such as neutropenic patients who are not receiving mould-active prophylaxis, where a negative result is reassuring in ruling out IA. In other settings including non-neutropenic patients and patients at low risk for IA, like GM the utility of PCR testing is limited, particularly from blood (Egger *et al.* 2020), and overall it suffers from poor precision, with a specificity of 76 % (Arvanitis *et al.* 2014). Performance of blood PCR is particularly poor in patients on mould-active prophylaxis (Egger *et al.* 2020).

Two point-of-care tests are now available for the diagnosis of IA. The *Aspergillus*-specific Lateral Flow Device (LFD) test (OLM Diagnostics, Newcastle Upon Tyne, United Kingdom) detects an extracellular mannoprotein antigen secreted exclusively during active growth of *Aspergillus* species via the JF5 monoclonal antibody (Hoenigl *et al.* 2014b, 2018, Prattes *et al.* 2015, Orasch *et al.* 2017). Another new test, the sona *Aspergillus* GM-LFA (IMMY, Norman, OK, United States) detects GM but has a shorter turnaround time compared to the conventional GM ELISA test. In patients with hematologic malignancy, both the LFD and LFA have a comparable sensitivity and specificity to GM from BALF for diagnosing IA (Heldt *et al.* 2018, de Heer *et al.* 2019, Jenks *et al.* 2019c, 2021, Mercier *et al.* 2020), and sensitivity increased further when combined with inflammatory markers, triacetylfusarinine C, or PCR (Hoenigl *et al.* 2019, Jenks *et al.* 2019d, 2019e, Rawlings *et al.* 2019). In non-neutropenic patients, the LFA and LFD have demonstrated a sensitivity and specificity between 60–70 % when used alone and up to 80 % when used in combination (Jenks *et al.* 2019b), with tendencies towards better performance of the LFA (Jenks *et al.* 2020). Particularly the LFA, but also the LFD have also shown promise for diagnosing IA in serum samples from patients with haematological malignancies (Jenks & Hoenigl 2020). Unmet needs for the diagnosis of IA include a true point-of-care test that can be done at the bedside or in the clinic in the matter of minutes. In addition, improved diagnostic algorithms to diagnose IA in non-neutropenic patients are needed as well.

The detection of *Aspergillus* serum precipitin antibodies (*i.e.*, subsets of IgG and IgM antibodies) is useful for the diagnosis of ABPA (Agarwal *et al.* 2013). The detection of *Aspergillus* IgG antibodies via commercial ELISAs or POC tests (Richardson & Page 2018) is the mainstay diagnostic test for aspergilloma (Hope *et al.* 2005) and chronic pulmonary aspergillosis (CPA) (Denning *et al.* 2016), when used in conjunction with pulmonary imaging, but *Aspergillus* IgG lacks the specificity in the diagnosis of IA (Richardson & Page 2017), and GM and also the LFD have limited sensitivity in those with CPA (Salzer *et al.* 2018). *Aspergillus* IgG are also present in *Aspergillus* bronchitis, *Aspergillus* nodule and chronic rhinosinusitis, and can be used for treatment stratification in CPA (Denning *et al.* 2018).

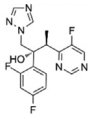
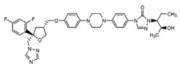
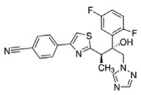
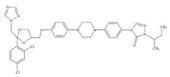
CLINICAL TREATMENT OF ASPERGILLOSIS

Triazoles, particularly voriconazole, isavuconazole and posaconazole for invasive infections, and voriconazole or itraconazole for chronic infections, are the first line antifungal agents used to treat aspergillosis (Denning *et al.* 2016, Cornely *et al.* 2019a, Jenks *et al.* 2019a). Voriconazole and isavuconazole exert fungicidal activity by inhibiting ergosterol biosynthesis. Ergosterol is one of the main structural components of the fungal cell membrane, allowing membrane fluidity, proper positioning and function of membrane-integrated proteins, and the cell cycle. Azoles bind and inhibit at the active site of the cytochrome P450 enzyme lanosterol 14-α-demethylase, which contains a heme cofactor and is encoded by two homologous genes, *CYP51A* and the *CYP51B* (a third homolog *CYP51C* has been found in *A. flavus*) (Dudakova *et al.* 2017). This induces the accumulation of 14-methyl sterols, such as lanosterol, which alters functions of cell membrane leading to fungal growth arrest. Furthermore, inhibition of ergosterol biosynthesis results in both accumulation of toxic sterol intermediates and creation of cell wall carbohydrate patches that extend to the plasma membrane (Geiβel *et al.* 2018). Drug-drug interactions may limit the use of voriconazole and other triazoles not only in the ICU setting, but also in patients with haematological malignancies where some newer drugs, including ibrutinib, venetoclax, and midostaurin, may complicate the use of triazoles (Groll *et al.* 2017, Tapaninen *et al.* 2020).

The therapeutic approach of IA has been changed with the development of the second-generation mould-active triazoles, voriconazole, posaconazole and isavuconazole in addition to the first-generation azole itraconazole. The chemical structure of voriconazole closely resembles fluconazole and shares a similarity with isavuconazole, while posaconazole more closely resembles itraconazole. Voriconazole was derived from fluconazole by replacing one triazole moiety in fluconazole with a 4-fluoropyrimidine group and adding an α-methyl group (Bellmann & Smuszkiewicz 2017). Posaconazole derives from itraconazole through the replacement of the chlorine substituents with flourine in the phenyl ring, as well as hydroxylation of the triazolone side chain (https://pubchem.ncbi.nlm.nih.gov/). Unlike the other second-generation triazoles, isavuconazole is administered as a prodrug; the isavuconazoilum sulfate ester which is hydrolysed rapidly by serum esterases, is highly water soluble and does not require the addition of a beta-cyclodextrin to facilitate solubility (Jenks *et al.* 2018).

The availability of both intravenous and different oral formulations of triazoles increases the therapeutic options and improves their pharmacokinetics (Table 1) (Cornely *et al.* 2019b). The variable pharmacokinetics (80–100 %) of voriconazole (oral

Table 1. Pharmacokinetic and pharmacodynamic properties of anti-*Aspergillus* azoles.

Parameters	Voriconazole	Posaconazole	Isavuconazole	Itraconazole
Chemical structure				
Molecular weight	349.3	700.8	717.77	705.64
Water solubility (mg/mL)	0.0978	0.012	0.0162	0.00964
Log D (pH 7.4)	1.8	2.15	4.14	> 5
Formulations	Oral solution, tablet, iv	Oral solution, tablet, iv	Capsules, iv	Oral solution, tablet, capsules, iv
Standard dose	LD: 6 mg/kg q12h, MD: 4 mg/kg q12h or 200 mg PO	Oral = 200 mg q6h/q8h or 400 mg q12h Tablet / iv = 300 mg q24h	LD: 200 mg q6h for 2 d; MD: 200 mg q24h	Oral = LD: 800 mg q12h/g24h for 2 d, MD: 200 mg q12h IV = LD: 200 mg q12h for 2d, MD: 200 mg q24h
Dose adjustment	< 40 kg = 50 % MD dose, RI = avoid iv[1], HI = 50 % MD, IR = 300mg q12h	RI = avoid iv[1]	No	RI = avoid iv[1]
Tmax	1.43–1.81	Oral = 3, tablet = 2.2, iv = 1.5	Oral = 2–3, iv = 0.75–1	Oral = 5, Iv = 1
Bioavailability	90 % (healthy) / 83 % (patients)	Oral = 8–47 %	98 %	Capsules = 22 %; oral solution = 55–92 %
Effect of food	22 % reduction	Oral = ↑ with high fat meal/low pH, tablet = ↓ mucositis, ↑ with high fat meal	No	Capsules = ↑ with high fat meal/low pH, oral solution = ↑ empty stomach
Protein binding	42–58 %	98–99 %	99 %	99.8 %
Vd (Lt/kg)	2–4.6	Oral = 3.7, tablet = 5, iv = 20	6.42	11
Tissue penetration	Brain, ELF	Alveolar cells, liver kidney, lung, myocardium	Muscle, fat, liver, brain	Skin, fat, liver, lung, kidney, spleen, bone, muscle
CL (mL/min/kg)	3–8.3	9.39–16.4 L/h	1.9–5	5.1
Hepatic/Urinary (metabolites and drug) excretion %	20 (M)+< 1(D) / 80 (M)+< 2(D)	77 (M)+< 5(D) / 14 (M)+< 5(D)	46 (M) / 46 (M)+0.38(D)	54 (M)+< 0.03(D) / 35 (M)+3–18(D)
Metabolizing enzymes	CYP2C19 > CYP2C9,CYP3A4	UGT1A4	CYP3A4, CYP3A5, UGT	CYP3A4, CYP2C9, CYP2C19
$T_{1/2}$ (h)	6–12	27–35	110–130	24
Cmax (mg/L)	4.84 (300 mg), 5.27 (400 mg)	Oral = 0.851, tablet = 1.96, iv = 3.28	2.6	0.5–2.3
AUC_{0-24} (mg*h/mL)	13.21–16.38	Oral = 17.24, tablet / iv = 34.3	98–121	29.2
Variation in AUC_{0-24}	82 %	Oral = 82 %, tablet / iv = 35–40 %	10–43 %	30–60 %
Pharmacokinetics	Non-linear (saturable metabolism)	Oral = non-linear (saturable absorption), tablet / iv = linear	Linear	Non-linear (saturable absorption and metabolism)
tAUC/MIC (50 % survival in animals)	17.61–21.96	167–178	25	NA
TDM targets (mg/L)	Prophylaxis: C_{min} > 0.5; therapy: C_{min} > 1–2; toxicity: C_{min} < 4–6	prophylaxis: C_{min} > 0.5–0.7; therapy: C_{min} > 1–1.25	NA	Prophylaxis: C_{min} > 0.5; therapy: C_{min} > 0.5–1; toxicity: C_{avg} < 17 (bioassay), < 3.5 (HPLC)

NA, not applicable.
[1] Unless an assessment of the benefit/risk to the patient justifies the use of intravenous formulations.

solution, tablet and intravenous [IV] formulation) and the oral solution of posaconazole due to erratic hepatic metabolism and absorption, respectively, have been improved with the new formulations of posaconazole i.v./tablet and isavuconazole i.v./capsules (~50 % variation) (Jović *et al.* 2019). The bioavailability of oral formulations ranges from poor with the "old" posaconazole oral solution to high with isavuconazole, and intake with fatty food is most important for the posaconazole oral solution (Hoenigl *et al.* 2014a). All four azoles exhibit non-linear pharmacokinetics because of saturable absorption or metabolism except isavuconazole (Bellmann & Smuszkiewicz 2017). They are highly protein bound (> 98 %) except voriconazole (58 %). All

azoles are characterised by a large volume of distributions (3–11 lt/kg) and they are extensively metabolised with minimal amounts of parent drug excreted renally or hepatobiliarily. Their half-lives and total drug exposure area under curve (AUC) varies from short with voriconazole (6–12 h and 13–16 mg*h/l) to long with isavuconazole (110–130 h and 98–121 mg*h/l, respectively) (Table 1). As will be discussed in detail later in this review, the emergence of ARAF isolates threatens the efficacy of azoles, and lipid formulations of amphotericin B, as well as echinocandins (which should be used in combination with another antifungal drug) are alternative treatment options for IA (Patterson *et al.* 2016). Surgery in addition to systemic antifungal therapy plays an important role in the treatment of primary extrapulmonary invasive aspergillosis (Aldosari *et al.* 2020).

Antifungal combination therapy with voriconazole or amphotericin B and an echinocandin is often employed as primary or salvage therapy for management particularly of refractory aspergillosis (Elefanti *et al.* 2013). Resistance to first line triazole antifungal agents among *Aspergillus* species has resulted in the increased use of second-line monotherapy with echinocandin drugs (caspofungin, micafungin or anidulafungin) (Aruanno *et al.* 2019). Echinocandin class drugs inhibit the cell wall biosynthetic enzyme β-(1,3)-d-glucan synthase (Perlin 2015), and were initially approved by the FDA for the treatment of invasive aspergillosis refractory to conventional therapy (Johnson & Perfect 2003). Given a strong 20-year history of safety and efficacy, it is being used increasingly in patients being treated for chronic pulmonary aspergillosis. A recent meta-analysis of 12 studies covering 380 patients who received IV antifungals, either amphotericin B (n = 143) or an echinocandin (n = 237) reported a response of 58 % for amphotericin B and 62 % for echinocandins (micafungin). Echinocandins, especially micafungin are well-tolerated and effective prophylactic antifungal agents used in patients with hematologic diseases at high risk for invasive mould infections (Ziakas *et al.* 2014, Park *et al.* 2019).

WORLDWIDE EMERGENCE OF AZOLE-RESISTANT *A. FUMIGATUS*: ENVIRONMENTAL AND CLINICAL ROUTES

Azole drugs play a major role in prevention and treatment of infections caused by *Aspergillus* species. Azole drugs belong to a wider group of compounds called demethylation inhibitors (DMIs) whose common target is the 14-α sterol demethylase. Demethylation inhibitors are widely used in the clinical setting, both as treatment and prophylaxis, but also in the agriculture setting due to their high-efficiency and broad-spectrum activity (Price *et al.* 2015, Hollomon 2017).

Several azole-based fungicides used in agriculture to protect cereals and wine from phytopathogenic moulds have a similar chemical structure to medical triazoles and the development of cross-resistance between them has been proved (Snelders *et al.* 2012, Zhang *et al.* 2017). A large number of demethylation inhibitor fungicides have been used intensively in agriculture and medicine (human and veterinary) since 1970 (Rochette *et al.* 2003). Though azole fungicides are not used to target *A. fumigatus*, it transpires that many of these fungicides are active against *A. fumigatus*, a condition that led to the emergence of resistance. More than 30 azole fungicides have been studied for their *in vitro* activity against *A. fumigatus*, including propiconazole, bromuconazole, epoxiconazole, difenconazole, and tebuconazole. In this context, one of the presumed routes for triazole-resistance emergence is through selection pressure from the DMIs used as fungicides in the environment (Verweij *et al.* 2007, 2009). The resulting azole resistant isolates are associated with a particular resistance mechanism constituted by a variable number of tandem repeat (TR) integrations in the *CYP51A* promoter followed or not by point mutations in the coding gene (Snelders *et al.* 2008). There is a lot of evidence that supports the idea of an environmental driven mechanism, such as the fact that these TR azole resistant strains (TR_{34}/L98H, TR_{34}/L98H/S297T/F495I, TR_{46}/Y121F/T289A, and TR_{53}) have been detected throughout the world (Garcia-Rubio *et al.* 2017) but showed shorter genetic distances among them than with other azole-susceptible and non TR-resistant isolates, which suggests that they could have developed from a reduced set of clonally related strains (Snelders *et al.* 2008, Camps *et al.* 2012b, Garcia-Rubio *et al.* 2018b). Besides, the fact that TR azole resistant strains have been isolated from azole-naïve patients with IA also endorses the existence of this environmental resistance route, indicating that infections would have been caused by the inhalation of already ARAF spores harbouring aforementioned mutations in *CYP51A* (Snelders *et al.* 2009, Verweij *et al.* 2009). However, other single mutations occurring in *CYP51A* arise during the course of azole therapy (Howard *et al.* 2006, 2009, Albarrag *et al.* 2011, Camps *et al.* 2012c, Wiederhold *et al.* 2016).

Nevertheless, there are also some findings that support that TR azole-resistance mechanisms do not seem to be restricted to the environment. A clinical case of fatal aspergillosis caused by an *A. fumigatus* strain that developed a TR_{120} insertion in the *CYP51A* promoter during long-term azole treatment has been recently reported using both STRA*f* typing and whole-genome sequencing (Hare *et al.* 2019). This challenges the existence of a link between resistance mechanisms and specific routes of resistance selection and may fade the presumed boundaries between the environmental and clinical routes of resistance. In line with these facts, strains carrying G432S and G432A mutations have been isolated from azole-naïve patients (Howard *et al.* 2006, 2009, Alanio *et al.* 2011, Albarrag *et al.* 2011), while strains carrying TR_{53} (and also TR_{120}) mutations have been isolated from patients exposed to azole antifungals in prior treatments (Hodiamont *et al.* 2009, Hare *et al.* 2019). Furthermore, studies about how these supposedly environmental resistance mechanisms originated are still scarce, although it has been hypothesised that environmental conditions or even a more complex reproductive method, such as sexual reproduction, could play a role. In addition, the dispersion of *A. fumigatus* spores from the human lung into the environment has been suggested lately as a possible transmission path in cystic fibrosis patients as an alternative transmission route from patient to environment (Engel *et al.* 2019). Although azole resistance may predominantly originate from environmental sources, further research is warranted in order to gain a deeper knowledge about how azole resistance emerges and is transmitted, which has implications for patient management.

Table 2. Diversity and prevalence of *CYP51A* mutations causing azole resistance in clinical *A. fumigatus* isolates.

		MIC values (μg/mL)			Azole exposure prior resistance		Azole therapeutic failure		
Country (year; number of isolates)	**Amino acid substitutions (total number) (references)**	**VRZ**	**PSZ**	**ITZ**	**Present**	**Naïve**	**VRZ**	**PSZ**	**ITZ**
the Netherlands (1994–2013; 186), Germany (2002–2018; 111), Italy (1995–2006, 2013–2015; 28), China (2008–2016; 19), Belgium (2011–2016; 18), Taiwan (2011–2018; 16), Denmark (2010–2014; 12), India (2008–2014; 11), UK (1992–2017; 11), USA (2001–2017; 7), Iran (2003–2016; 5), Tanzania (2016; 5), France (2012; 3), Spain (2014–2018; 3), Brazil (2014–2018; 3), Pakistan (2016; 3), Japan (2016–2018; 3), Switzerland (2016; 2), Portugal (2010–2016; 2), Kuwait (2015; 2), Mexico (2014–2017; 2), Australia (2015–2017; 1)	TR_{34} / L98H (T297S / F495I) (453) (Mellado *et al.* 2007, Verweij *et al.* 2007, Snelders *et al.* 2008, Howard *et al.* 2009, Lockhart *et al.* 2011, van der Linden *et al.* 2011, 2013, Rath *et al.* 2012, Seyedmousavi *et al.* 2013b, Ahmad *et al.* 2015, Chowdhary *et al.* 2015, Liu *et al.* 2015, Vermeulen *et al.* 2015, Wu *et al.* 2015, 2020, Chen *et al.* 2016b, Jensen *et al.* 2016, Lazzarini *et al.* 2016, Mushi *et al.* 2016, Nabili *et al.* 2016, Perveen *et al.* 2016, Wiederhold *et al.* 2016, Koehler *et al.* 2017, Montesinos *et al.* 2017, Prigitano *et al.* 2017, Abdolrasouli *et al.* 2018b, Berkow *et al.* 2018, Denardi *et al.* 2018, Pinto *et al.* 2018, Riat *et al.* 2018, Seufert *et al.* 2018, Talbot *et al.* 2018, Gonzalez-Lara *et al.* 2019, Rivero-Menendez *et al.* 2019b, Tsuchido *et al.* 2019, Pontes *et al.* 2020)	0.5–>16	0.125–>8	>4–32	Yes FLZ VRZ ITZ PSZ	Yes	Yes	Yes	Yes
the Netherlands (2009–2013; 29), Belgium (2011–2016; 11), Denmark (2010–2014; 6), Germany (2015–2018; 3), USA (2001–2016; 3), China (2010–2016; 3), France (2014; 2), Spain (2014–2018; 2), UK (2016; 1), Portugal (2010–2016; 1), Argentina (2009; 1), Japan (2013; 1), Taiwan (2011–2018; 1)	TR_{46} / Y121F / T289A (64) (Verweij *et al.* 1998, Kuipers *et al.* 2011, Lockhart *et al.* 2011, Vermeulen *et al.* 2012, 2015, Montesinos *et al.* 2013, van der Linden *et al.* 2011, 2013, Astvad *et al.* 2014, Chen *et al.* 2015, 2016b, Peláez *et al.* 2015, Steinmann *et al.* 2015, Jensen *et al.* 2016, Vazquez & Manavathu 2016, Wiederhold *et al.* 2016, Lavergne *et al.* 2017, Moore *et al.* 2017, Isla *et al.* 2018, Pinto *et al.* 2018, Seufert *et al.* 2018, Rivero-Menendez *et al.* 2019b, Li *et al.* 2020, Wu *et al.* 2020)	>8–>32	0.125–2	1–>16	ITZ, VRZ PSZ FLZ	Yes	Yes	Yes	Yes
The Netherlands (2009; 1)	TR_{53} (1) (Hodiamont *et al.* 2009)	16	0.5	16	ITZ		Yes/No	No	Yes
Denmark (2016; 1)	TR_{120} (1) (Hare *et al.* 2019)	4	0.5	16–>16	Yes VRZ PSZ		Yes	Yes	ND
Germany (2018; 8), Japan (2012; 8), Italy (1995–2006; 7), UK (1992–2009; 6), USA (2001–2014; 5), China (2001; 3), Australia (2015–2017; 2), the Netherlands (2007–2009; 2), India (2011–2014; 2), Spain (2014–2018; 2), Denmark (2010–2014; 1)	G54R/E/W (46) (Chen *et al.* 2005, Bueid *et al.* 2010, van der Linden *et al.* 2011, Camps *et al.* 2012c, Tashiro *et al.* 2012, Chowdhary *et al.* 2015, Wiederhold *et al.* 2015, 2016, Jensen *et al.* 2016, Lazzarini *et al.* 2016, Talbot *et al.* 2018, Rivero-Menendez *et al.* 2019b)	0.125–4	1–>16	1–>16	Yes ITZ PSZ VRZ	ND	Yes	ND	Yes
UK (1992–2007; 16), USA (2001–2014; 2)	G138C/S (18) (Howard *et al.* 2006, 2009, Albarrag *et al.* 2011, Wiederhold *et al.* 2016)	16–8	1–4	>8–16	Yes ITZ VRZ	ND	Yes	ND	Yes

Table 2. (Continued).

Country (year; number of isolates)	Amino acid substitutions (total number) (references)	MIC values (μg/mL) VRZ	PSZ	ITZ	Azole exposure prior resistance Present	Naïve	Azole therapeutic failure VRZ	PSZ	ITZ
The Netherlands (2007–2009; 3), India (2012–2016; 3), Denmark (2010–2014; 2), UK (1992–2009; 2), USA (2015–2017; 1), Japan (2009–2011; 1)	P216L (12) (Howard *et al.* 2009, Bueid *et al.* 2010, Camps *et al.* 2012c, Hagiwara *et al.* 2014, Ahmad *et al.* 2015, Jensen *et al.* 2016, Berkow *et al.* 2018, Dabas *et al.* 2018)	0.25–2	0.06–2	2–>16	ND	ND	ND	ND	ND
The Netherlands (2007–2009; 8), USA (2001–2014; 1), Italy (2013–2015; 1)	F219I/S/L (10) (van der Linden *et al.* 2011, Wiederhold *et al.* 2016, Prigitano *et al.* 2017)	0.25–8	0.25–>16	>16	Yes ITZ	ND	Yes	Yes	Yes
UK (1992–2009; 10), USA (2001–2014; 4), the Netherlands (1994–2007; 1), Denmark (2010–2014; 1), Sweden (1997–1998; 1), Japan (2017–2018; 1), China (2001; 1)	M220V/K/I/R/W (19) (Chen *et al.* 2005, Snelders *et al.* 2008, Howard *et al.* 2009, Bueid *et al.* 2010, Jensen *et al.* 2016, Wiederhold *et al.* 2016, Dabas *et al.* 2018, Tsuchido *et al.* 2019)	0.5–4	0.5–>4	>4–>16	Yes ITZ				ITZ
Japan (2000; 1)	F332K (Asano *et al.* 2011)	0.25	0.5	32	ND	ND	ND	ND	ND
USA (2001–2014; 4), UK (1992–2009; 3), Belgium (2015–2016; 2), Spain (2011; 1), Japan (2017–2018; 1), France (2006; 1)	G448S (12) (Howard *et al.* 2009, Bellete *et al.* 2010, Bueid *et al.* 2010, Peláez *et al.* 2012, Wiederhold *et al.* 2016, Montesinos *et al.* 2017)	0.5–>16	0.25–4	0.5–>16	Yes ITZ VRZ	ND	Yes	ND	Yes
UK (1992–2007; 2), India (2012–2016; 2)	Y431C (4) (Howard *et al.* 2006, 2009, Albarrag *et al.* 2011, Dabas *et al.* 2018)	0.5–4	0.06–2	>8–16	ND	ND	ND	ND	ND
China (2011–2014; 1), France (2006–2009; 1)	G432A/S (2) (Alanio *et al.* 2011, Liu *et al.* 2015)	0.25–2	0.25–0.5	4–16	ND	ND	ND	ND	ND
UK (1992–2007; 2)	G434C (2) (Howard *et al.* 2006, 2009, Albarrag *et al.* 2011)	4	1	>8	No	Yes	ND	ND	ND
UK (1992–2017; 70),# China (2008–2009; 16), Spain (2014–2018; 17), Japan (2009–2018; 8), Denmark (2010–2014;7), USA (2015–2017; 7), Italy (1995–2006, 2013–2015; 6), Taiwan (2011–2018; 6), the Netherlands (1994–2013; 4), Belgium (2011–2016; 4), Portugal (2008–2016; 4), Sweden (1997–1998; 2), South Korea (2012–2013; 2), Iran (2003–2016; 2), Brazil (2008–2009; 1), Czech Republic (2008–2009; 1), Germany (2015; 1)	WT (158) (Chryssanthou 1997, Snelders *et al.* 2008, Bueid *et al.* 2010, Lockhart *et al.* 2011, Tashiro *et al.* 2012, Hagiwara *et al.* 2014, Fuhren *et al.* 2015, Steinmann *et al.* 2015, Vermeulen *et al.* 2015, Wiederhold *et al.* 2015, 2016, Jensen *et al.* 2016, Lazzarini *et al.* 2016, Nabili *et al.* 2016, Lavergne *et al.* 2017, Montesinos *et al.* 2017, Prigitano *et al.* 2017, Abdolrasouli *et al.* 2018b, Berkow *et al.* 2018, Isla *et al.* 2018, Pinto *et al.* 2018, Seufert *et al.* 2018, Tsuchido *et al.* 2019, Won *et al.* 2020)	0.25–16	0.06–16	0.5–>16	ITZ	ND	ND	ND	Yes

MIC, minimum inhibitory concentration; FLZ, fluconazole; ITZ, itraconazole; PSZ, posaconazole; VRZ, voriconazole.

THE WORLDWIDE CLINICAL BURDEN OF ARAF

The increasing burden of azole resistance on a global scale notoriously limits the therapeutic options to treat aspergillosis (Denning & Perlin 2011). Over the last two decades, a rapid local and global emergence of triazole resistance has been observed. The first ARAF isolates were obtained from two patients treated with itraconazole, one of whom died early 1990, in California, in a case that dates back to the late 1980s (Denning *et al.* 1997b). A Dutch study later reported three ARAF isolates recovered after long-term itraconazole treatment from a lung transplant recipient in 1997 (Verweij *et al.* 2002). Moreover, a study in France found four itraconazole-resistant isolates with high itraconazole minimum inhibitory concentrations (MICs) values, > 16 mg/L, in 1999 (Dannaoui *et al.* 1999b). Later, in 2007, a comprehensive study of nine cases of azole-resistant IA found that four out of nine patients had never previously been treated with azole antifungals (Hussain *et al.* 2007).

Studies have investigated the distribution of ARAF in relation to the TR_{34}/L98H mutation, which was first found in the

Table 3. Diversity and prevalence of *CYP51A* mutations causing azole resistance in environmental *A. fumigatus* isolates.

	MIC[1] values (mg/L)				Azole exposure prior resistance		
Country (year of isolation; number of isolation)	**Amino acid substitutions (total number) (references)**	**VRZ**	**PSZ**	**ITZ**	**Present**	**Naive**	**Source of isolate**
The Netherlands (2002–2019; 203); Denmark (2009; 4); India (2011–2014; 56); Iran (2013; 5); China (200–2015; 4); Thailand (2014–2015; 8); Italy (2011–2018; 61); Romania (2013–2014; 16);UK (2009–2018; 43); France (2010–2017; 62); Taiwan (2014–2018; 33); Tanzania (2013–2014; 11); Australia (2013–2017; 1); Kuwait (2013–2015; 9); USA (2015; 38); Germany (2012–2013; 45); Colombia (2015; 2)	TR_{34}/L98H (S297T/F495I/Q141H/S52T) (601) (Verweij *et al.* 2009, Mortensen *et al.* 2010, Chowdhary *et al.* 2012b, 2014a, 2014b, Badali *et al.* 2013, Ahmad *et al.* 2014, 2015, Bromley *et al.* 2014, Prigitano *et al.* 2014, Wang *et al.* 2014, Bader *et al.* 2015, Sharma *et al.* 2015, Chen *et al.* 2016b, 2019b, Nabili *et al.* 2016, van der Linden *et al.* 2016, Álvarez-Moreno *et al.* 2017, 2019, Hurst *et al.* 2017, Jeanvoine *et al.* 2017, Lavergne *et al.* 2017, Tangwattanachuleeporn *et al.* 2017, Rocchi *et al.* 2018, Talbot *et al.* 2018, Trovato *et al.* 2018, Tsitsopoulou *et al.* 2018, Sewell *et al.* 2019, Ahangarkani *et al.* 2020)	0.5–>32	0.25–3	>4–>16	Yes Bromuconazole, cyproconazole, difenoconazole, epoxiconazole, hexaconazole, metconazole, penconazole, propiconazole, tebuconazole, tricyclazole, triticonazole	No/Yes	Air, patient room, water filter, soil, corps, seeds, rose pot compost, compost. Hospital garden, cucurbit fields
Iran (2017–2018; 3); The Netherlands (2009–2011; 14); UK (2018; 6); Tanzania (2013–2014; 4); France (2014–2017; 31); Colombia (2015–2018; 38); India (2012–2013; 6); Germany (2012–2013; 6); Taiwan (2016–2018; 3); Greece (2016–2017; 1)	TR_{46} / Y121F / T289A (112) (van der Linden *et al.* 2013, 2016, Chowdhary *et al.* 2014a, 2014b, Bader *et al.* 2015, Sharma *et al.* 2015, Álvarez-Moreno *et al.* 2017, 2019, Lavergne *et al.* 2017, Rocchi *et al.* 2018, Chen *et al.* 2019b, Sewell *et al.* 2019, Siopi *et al.* 2020)	>2–≥16	0.25–2	>8–>16	Yes	No/Yes	Air, patient room, water filter, soil, corps, seeds, rose pot compost, compost, raisins, grapes. Hospital garden, cucurbit fields, farmland
India (2013–2014; 1); Romania (2013–2014; 7); Tanzania (2013–2014; 13); Australia (2013–2017; 2); Germany (2012–2013; 1); Thailand (2014–2015; 2)	G54R/E/W/A (26) (Bader *et al.* 2015, Sharma *et al.* 2015, Tangwattanachuleeporn *et al.* 2017, Talbot *et al.* 2018)	0.25–>32	0.25–2	16–>32	Yes Bromuconazole, cyproconazole, difenoconazole, epoxiconazole, hexaconazole, metconazole, penconazole, tebuconazole, tricyclazole, triticonazole	ND	Hospital soil, woody debris of trunk hollows, garden soil
Australia (2013–2017; 41); Germany (2012–2013; 1); Taiwan (2014–2016; 5); Colombia (2015–2018; 5)	WT(52) (Bader *et al.* 2015, Álvarez-Moreno *et al.* 2017, 2019, Talbot *et al.* 2018, Wang *et al.* 2018b)	1–>32	0.125–1	0.25–>32	Yes	No/Yes	Azole-naïve and azole exposed soil, air
Italy (2011-2012; 1) Australia (2013-2017; 1); Germany (2012–2013; 1)		1–>32	0.5–2	>16–>32	Yes	ND	Cucurbit fields

Table 3. (Continued).

Country (year of isolation; number of isolation)	MIC[1] values (mg/L)				Azole exposure prior resistance		Source of isolate
	Amino acid substitutions (total number) (references)	VRZ	PSZ	ITZ	Present	Naive	
	F46Y; M172I/V; N248K/T; D255E (3) (Prigitano *et al.* 2014, Bader *et al.* 2015, Talbot *et al.* 2018)						
France (2014–2016; 20)	P216L (Lazzarini *et al.* 2016, Jeanvoine *et al.* 2017)	0.5	0.5	>16	Yes	ND	Sawmills and air
Germany (2012–2013; 1)	M220I (Bader *et al.* 2015)	1–>32	0.125–1	0.125–>32	ND	ND	Soils
Colombia (2015–2018; 4)	TR_{53} (van der Linden *et al.* 2016, Álvarez-Moreno *et al.* 2017, 2019)	4	ND	4–>8	Yes	No	Flower fields, soil and greenhouse soil

[1] MIC, minimum inhibitory concentration; ITZ, itraconazole; PSZ, posaconazole; VRZ, voriconazole.

Netherlands in 1998 (Snelders *et al.* 2008). Indeed, surveillance studies and case series recently reported the global presence of ARAF harbouring TR_{34}/L98H, with reports from Europe, the Middle East, including Australia, Tanzania, Kuwait and Iran, North and South Asia, including China and Japan, Australia and Tanzania; Africa, and North and South America, including Brazil and Columbia (Mellado *et al.* 2007, Verweij *et al.* 2007, Snelders *et al.* 2008, Howard *et al.* 2009, Lockhart *et al.* 2011, van der Linden *et al.* 2011, 2013, Chowdhary *et al.* 2012a, 2015, Rath *et al.* 2012, Seyedmousavi *et al.* 2013b, Astvad *et al.* 2014, Ahmad *et al.* 2015, Choukri *et al.* 2015, Fuhren *et al.* 2015, Liu *et al.* 2015, Steinmann *et al.* 2015, Vermeulen *et al.* 2015, Wu *et al.* 2015, 2020, Chen *et al.* 2016b, Jensen *et al.* 2016, Lazzarini *et al.* 2016, Mushi *et al.* 2016, Nabili *et al.* 2016, Perveen *et al.* 2016, Wiederhold *et al.* 2016, Koehler *et al.* 2017, Montesinos *et al.* 2017, Prigitano *et al.* 2017, Toyotome *et al.* 2017, Abdolrasouli *et al.* 2018a, 2018b, Berkow *et al.* 2018, Denardi *et al.* 2018, Pinto *et al.* 2018, Riat *et al.* 2018, Seufert *et al.* 2018, Talbot *et al.* 2018, Gonzalez-Lara *et al.* 2019, Rivero-Menendez *et al.* 2019b, Tsuchido *et al.* 2019, Li *et al.* 2020, Pontes *et al.* 2020) (Table 2). These studies describe the most recent discoveries of the TR_{46}/Y121F/T289A resistance mechanism involving environmental mutations, which the Netherlands first reported in 2009 (van der Linden *et al.* 2013); another report revealed that a US patient was recovering from TR_{46}/Y121F/T289A *A. fumigatus* infection in 2008 (Wiederhold *et al.* 2015).

In the following years, many more studies have been reported, from Argentina, China, Europe (Portugal, Spain, UK), Japan, Taiwan and US (Table 2) (Lockhart *et al.* 2011, van der Linden *et al.* 2013, Astvad *et al.* 2014, Steinmann *et al.* 2015, Vermeulen *et al.* 2015, Chen *et al.* 2016b, Jensen *et al.* 2016, Wiederhold *et al.* 2016, Lavergne *et al.* 2017, Montesinos *et al.* 2017, Isla *et al.* 2018, Pinto *et al.* 2018, Seufert *et al.* 2018, Li *et al.* 2020, Wu *et al.* 2020). Wild-type isolates have developed resistance to itraconazole in many countries and continents, such as the UK and the USA and in Europe and South Asia (Chryssanthou 1997, Snelders *et al.* 2008, Bueid *et al.* 2010, Lockhart *et al.* 2011, Tashiro *et al.* 2012, van der Linden *et al.* 2013, Hagiwara *et al.* 2014, Fuhren *et al.* 2015, Steinmann *et al.* 2015, Vermeulen *et al.* 2015, Jensen *et al.* 2016, Lazzarini *et al.* 2016, Nabili *et al.* 2016, Wiederhold *et al.* 2016, Prigitano *et al.* 2017, Abdolrasouli *et al.* 2018b, Berkow *et al.* 2018, Seufert *et al.* 2018). Other single nucleotide polymorphisms, in gene positions G54, M220, and G448 of the *CYP51A* gene, have been reported to be frequently observed in patients with chronic pulmonary aspergillosis, Invasive aspergillosis bronchitis, aspergilloma, and chronic cavitary pulmonary aspergillosis (CCPA) treated long term with azole antifungals, as well as several clinical treatment failures (Chryssanthou 1997, Chen *et al.* 2005, 2016b, Snelders *et al.* 2008, Howard *et al.* 2009, Bellete *et al.* 2010, van der Linden *et al.* 2011, Camps *et al.* 2012c, Tashiro *et al.* 2012, Chowdhary *et al.* 2015, Wiederhold *et al.* 2016, Montesinos *et al.* 2017, Dabas *et al.* 2018, Denardi *et al.* 2018, Riat *et al.* 2018, Seufert *et al.* 2018, Talbot *et al.* 2018, Tsuchido *et al.* 2019). Moreover, other single point mutations associated with resistance to azole antifungals have also been reported: G138C, F219I, P216L, G432S, and G432A (Howard *et al.* 2006, 2009, Bueid *et al.* 2010, Albarrag *et al.* 2011, van der Linden *et al.* 2011, Camps *et al.* 2012c, Hagiwara *et al.* 2014, Jensen *et al.* 2016, Wiederhold *et al.* 2016, Berkow *et al.* 2018, Dabas *et al.* 2018) (Table 2).

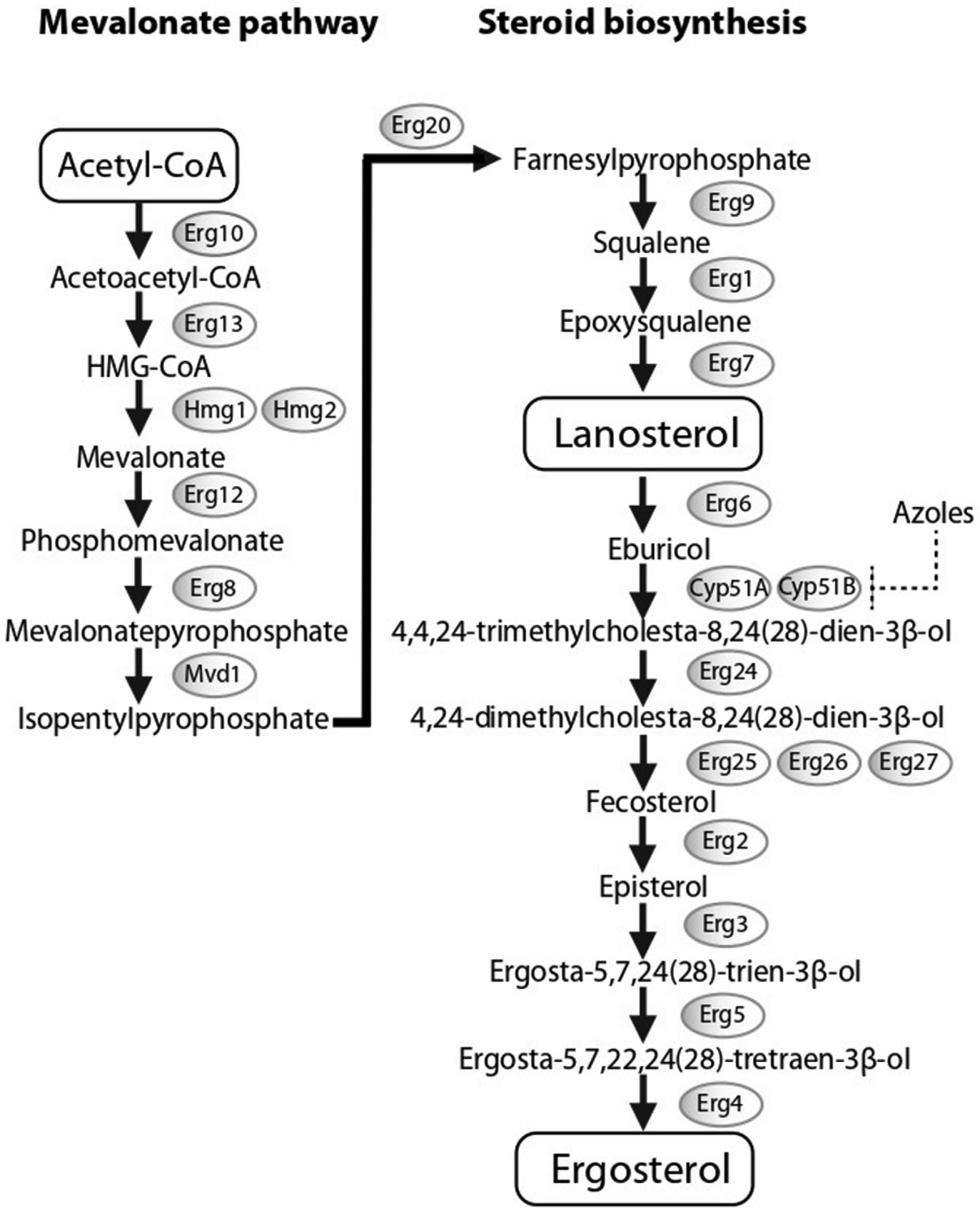


Fig. 2. The ergosterol pathway involves multiple enzymes and is tightly regulated by the key rate-limiting enzyme Hmg1 producing mevalonate. Lanosterol produced by ERG6 is the substrate catalysed by CYP51A and CYP51B, while azoles competitively occupy the catalytic site and hence reduce the ergosterol synthesis. Adopted from Moreno-Velásquez *et al.* (2017), with permission.

While matched control studies involving patients infected with azole-resistant and/or azole-susceptible isolates have not been conducted, patients with azole-resistant *Aspergillus* infections are at high risk for therapeutic failure. In the Netherlands surveillance study, a high mortality rate was reported among culture-positive ICU patients with ARAF; 14 patients, 10 of whom died, were identified with azole-resistant IA and several underlying conditions, such as autoimmune hepatitis, allogeneic stem cell transplant, hematologic malignancy non-small cell lung cancer, and chronic obstructive pulmonary diseases (COPD) (van

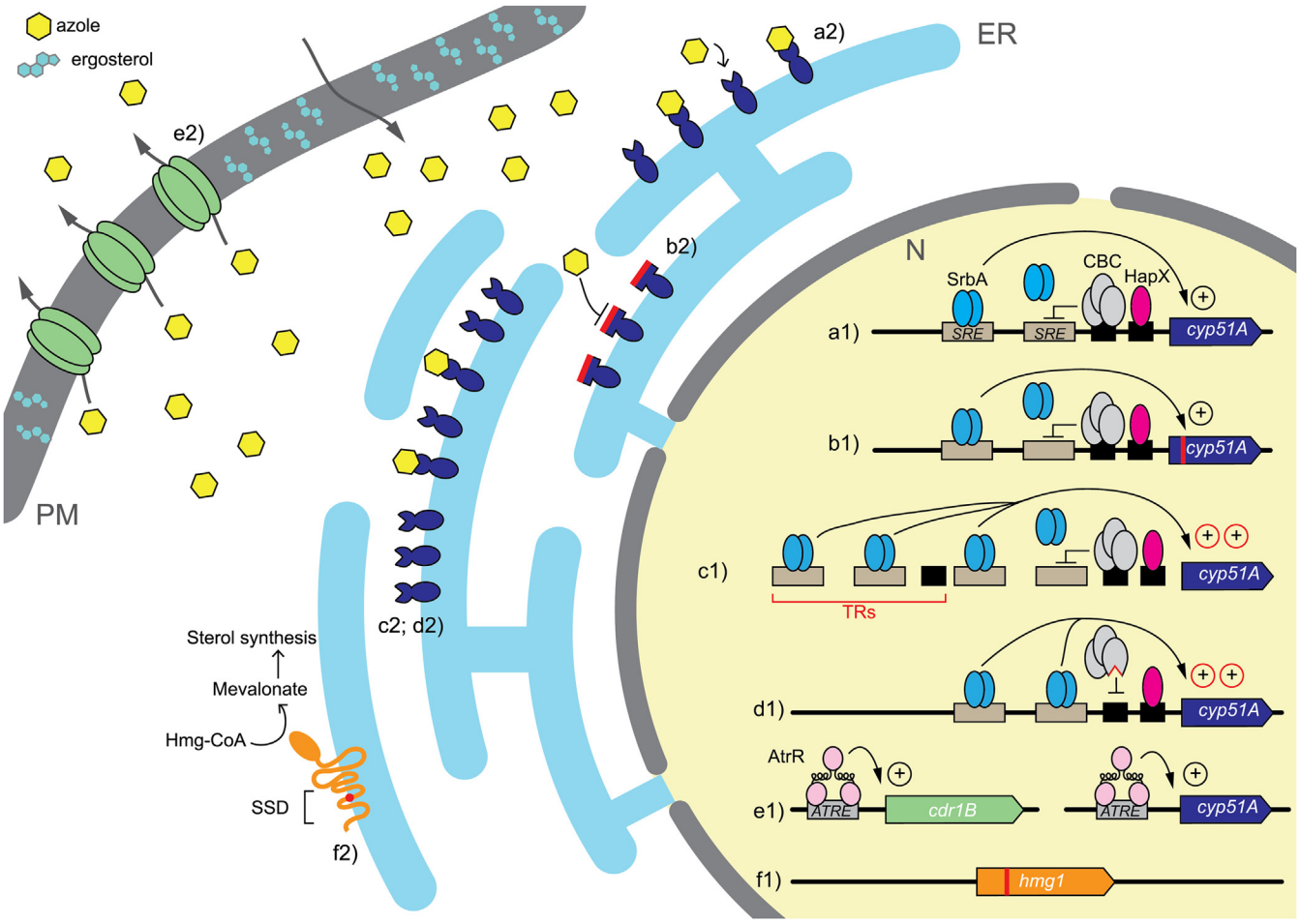


Fig. 3. Molecular mechanisms contributing to triazole resistance observed in *A. fumigatus*. The scheme represents an *A. fumigatus* cell - with particular focus on the nucleus (N), the endoplasmic reticulum (ER), and the plasma membrane (PM), which depicts the most relevant mechanisms of triazole resistance in this fungus. In *A. fumigatus*, a major role in ergosterol biosynthesis is played by the sterol demethylase CYP51A (a1). The *CYP51A* gene is regulated positively by the SrbA protein, which activates its expression by binding to two Sterol Regulatory Elements (SRE) in the promoter region. When ergosterol biosynthesis is repressed, the access of SrbA to SREs is prevented by both the CBC complex and the HapX transcription factor binding to regulatory elements located downstream of SREs, resulting in negative regulation of *CYP51A* expression. The sterol demethylase CYP51A, whose native substrate is eburicol, an intermediate of ergosterol biosynthesis, is the target of azole drugs (a2). As a result, changes in *CYP51A* sequence or expression are associated with increased MIC to triazoles. Amino acid substitutions in either the ligand binding site or the catalytic site (b1) modulates triazole binding affinity to CYP51A (b2). A different mutation that can be found in combination with SNPs in the *CYP51A* gene is the presence of tandem repeats (TRs) in the promoter region, resulting in an expansion of the SREs, unimpeded SrbA binding, and ultimately hyper-activation of *CYP51A* expression (c1). The same outcome was observed in the case of the P88L mutation in the HapE subunit of the CCAAT-binding complex (CBC) complex, which diminishes CBC binding affinity and its negative regulation of *CYP51A* expression, although this genotype had only been observed in the clinical isolates in which it was first described (d1). In both cases, the increased amount of the CYP51A enzyme prevents saturation by triazoles and sustains ergosterol biosynthesis (c2 and d2). As for other pathogenic fungi, overexpression of either ATP-binding cassettes (ABC) or Major Facilitator Superfamily (MFS) type drug efflux pumps had been observed among triazole-resistant clinical isolates, which prevents the accumulation of active concentration of drug in the cell. In particular, the transcription factor AtrR positively regulates the expression of the ABC transporter *CDR1B* (d1 and d2). Notably, AtrR is also involved in the positive regulation of *CYP51A*. A clinically relevant mutation of a different kind is the one affecting the Hmg-CoA reductase encoded by *hmg1*, which takes part in ergosterol biosynthesis by converting Hmg-CoA into Mevalonate. Hmg1 has a conserved Sterol Sensing Domain (SSD) involved in regulation of sterol biosynthesis. Mutations in the SSD result in a dysregulation of the sterol pathway that eventually translates to an increased cellular ergosterol production and triazole resistance (f1 and f2).

Paassen *et al.* 2016). Two patients died in Belgium of IA that progressed to cerebrospinal aspergillosis (Vermeulen *et al.* 2012). Other cases support these findings, including a recent German study in which seven of eight azole-resistant IA patients experienced failed therapeutic treatment and died (Salsé *et al.* 2019). It has also been shown that CPA patients have failed azole treatment due to azole resistance (Howard *et al.* 2009, Steinmann *et al.* 2015), while several other case series reported mortality rates of 50–100 % in patients with triazole-resistant IA. Resistance rates as high as 29 % have been observed in specific patient populations, such as critically ill patients (van Paassen *et al.* 2016, Verweij *et al.* 2016a).

WORLDWIDE BURDEN OF ARAF IN THE ENVIRONMENT

In recent years, an increasing proportion of *A. fumigatus* isolates has been observed to be resistant in patients (Table 2) and environments (Table 3) due to the presence of mutations in the *CYP51A* gene (Snelders *et al.* 2008). Numerous fungicides were able to inhibit wild-type strains, and some azole fungicides were active against wild-type strains but ineffective against isolates with the TR_{34}/L98H mutations, which have high MICs. In the Netherlands, these fungicides were introduced between 1990 and 1996, just before the first clinical TR_{34}/L98H strain was found

in 1998 (Snelders *et al.* 2008). Indeed, evidence of an ARAF environmental route of acquisition was first found in the Netherlands (Zhang *et al.* 2017). As a result, it has become evident that azole resistance has a potentially global distribution and is therefore a global problem (Mortensen *et al.* 2010, Verweij *et al.* 2016a).

Such environmental route-related mutations are present in many geographically diverse countries and continents (Table 3). Many countries have reported environmental ARAF isolates harbouring TR_{34}/L98H, including Australia, China, Colombia, Denmark, France, Germany, India, Iran, Italy, Kuwait, the Netherlands, Romania, Taiwan, Tanzania, Thailand, UK and the US (Verweij *et al.* 2009, Mortensen *et al.* 2010, Chowdhary *et al.* 2012b, 2014a, Badali *et al.* 2013, van der Linden *et al.* 2013, Ahmad *et al.* 2014, 2015, Bromley *et al.* 2014, Prigitano *et al.* 2014, Wang *et al.* 2014, Bader *et al.* 2015, Sharma *et al.* 2015, Chen *et al.* 2016b, Nabili *et al.* 2016, van der Linden *et al.* 2016, Álvarez-Moreno *et al.* 2017, Hurst *et al.* 2017, Jeanvoine *et al.* 2017, Tangwattanachuleeporn *et al.* 2017, Rocchi *et al.* 2018, Talbot *et al.* 2018, Trovato *et al.* 2018, Tsitsopoulou *et al.* 2018, Chen *et al.* 2019b, Sewell *et al.* 2019, Ahangarkani *et al.* 2020) (Table 3). Additionally, several studies have reported ARAF isolates harbouring TR_{46}/Y121F/T289A in Iran, the Netherlands, the UK, Tanzania, France, Colombia, India, Germany, and Taiwan (Chowdhary *et al.* 2014a, 2014b, Bader *et al.* 2015, Sharma *et al.* 2015, Nabili *et al.* 2016, van der Linden *et al.* 2016, Álvarez-Moreno *et al.* 2017, Lavergne *et al.* 2017, Rocchi *et al.* 2018, Chen *et al.* 2019b, Sewell *et al.* 2019). Moreover, wild-type strains have been reported in several countries, including Australia, Taiwan, Germany, and Colombia, for which the MICs values of itraconazole, voriconazole, and posaconazole ranged from 0.125 to > 32 (Bader *et al.* 2015, Álvarez-Moreno *et al.* 2017, 2019, Wang *et al.* 2018b).

Increasingly, other point mutations in azole-resistant strains have been reported in studies in Europe, North America, and Asia (Prigitano *et al.* 2014, Bader *et al.* 2015, Sharma *et al.* 2015, van der Linden *et al.* 2016, Álvarez-Moreno *et al.* 2017, 2019, Jeanvoine *et al.* 2017, Tangwattanachuleeporn *et al.* 2017, Talbot *et al.* 2018). Therefore, it is crucial to change processing practices to reduce the use and spread of azole fungicides in the environment that result in cross-resistance with medical azoles. Nonetheless, while TR_{34}/L98H and TR_{46}/Y121F/T289A mutations are currently the most prevalent mutations associated with the environmental route, ARAF strains without the *CYP51A* gene mutations may also emerge from the environment (Table 3). Indeed, the evidence demonstrates that environmental azole-resistance is increasing due to azole fungicide drugs in the environment; further study is needed.

ANTIFUNGAL TOLERANCE IN *A. FUMIGATUS*

Antifungal tolerance is different from antifungal resistance and a relatively new concept for medical mycology. Among the human pathogenic fungi, antifungal tolerance has been mainly studied in *Candida albicans* and *Candida glabrata*. Tolerance is defined as the ability of a subpopulation of cells (> 1 %) in a drug-susceptible strain to persist or grow in presence of drug concentrations above the minimal inhibitory concentration (MIC), often resulting in "trailing growth" in MIC assays. This is in contrast to heteroresistance or persistence, where very rare cells (< 1 %) grow above the MIC (Delarze & Sanglard 2015, Berman & Krysan 2020). *Candida albicans* tolerance is most commonly seen with azole antifungals and is quantified by disk diffusion or broth microdilution assays; in *C. glabrata* tolerance occurs readily with echinocandins. Tolerance is affected by strain genetics, especially chromosomal rearrangements or mutations in genes that participate in core stress pathways; it is also influenced by differences in growth conditions such as pH, temperature and nutrient availability and in physiological differences between genetically identical cells (Berman & Krysan 2020). The clinical relevance of antifungal tolerance in *Candida* species is still unresolved, with some studies failing to find a connection (Rueda *et al.* 2017), whereas others show a positive correlation between tolerance and disease persistence (Astvad *et al.* 2018, Healey & Perlin 2018, Rosenberg *et al.* 2018).

In *A. fumigatus*, tolerance has been best characterised for the echinocandin antifungals: micafungin, anidulafungin and especially caspofungin, which inhibit fungal β-1,3-d-glucan synthase activity, thereby disrupting cell wall integrity (Patil & Majumdar 2017). Echinocandin tolerance is defined by partial growth inhibition or trailing, under increasing drug concentrations, followed by an unusual phenomenon called the "paradoxical effect", during which hyphal growth intensifies despite increasing drug concentration (Wagener & Loiko 2017) until complete inhibition (MIC) at very high drug levels. Growth inhibition is characterised by the formation of stubby, highly budded compact colonies in which the growing hyphae undergo tip lysis followed by regenerative intrahyphal growth initiating from viable internal compartments (Moreno-Velásquez *et al.* 2017), with the minimal effective concentration (MEC) defined as the lowest drug concentration that induces compact colony formation. The mechanism(s) underlying *A. fumigatus* tolerance and paradoxical growth are not fully understood. A factor in overcoming echinocandin stress is the upregulation of chitin synthesis and a large increase in cell wall chitin levels (Walker *et al.* 2015). Three signalling pathways, the high osmolarity glycerol (HOG), HSP90/calcineurin and cell wall integrity pathway, upregulate chitin synthesis in response to echinocandin treatment (Wagener & Loiko 2017). Genetic or pharmacological targeting of these pathways blocked the upregulation of chitin synthesis and abolished *A. fumigatus* tolerance and paradoxical growth. Another important event that occurs after sustained echinocandin exposure is the relocalisation of glucan synthase from the vacuoles to the hyphal tips (Moreno-Velásquez *et al.* 2017). This is accompanied by increased cell wall β-1,3-d-glucan, and a reduction in chitin (Loiko & Wagener 2017). In animal models, increased drug levels elevate fungal burden but have no effect on overall survival (Wagener & Loiko 2017). Compact fragmented colonies are seen by histology, which may increase CFU counts while decreasing tissue penetration, organ injury and mortality (Petraitiene *et al.* 2002). Thus, the clinical relevance of echinocandin tolerance and the paradoxical effect is not yet clear.

In contrast to the literature on *Candida* spp., very few reports describe azole tolerance in aspergilli. Trailing reported for some clinical isolates of *A. flavus* and *A. niger* was dependent on inoculum size and growth medium, respectively (Mosquera *et al.* 2001, Wang *et al.* 2018a). Trailing also was described for *A. fumigatus* exposed to the allylamine antifungal terbinafine (Moore *et al.* 2001). No trailing growth has been reported with amphotericin B exposure. Recently, an important new

mechanism for tolerance and clinical resistance to echinocandins emerged in *A. fumigatus* in which it was reported that caspofungin induces a change in the membrane sphingolipid content rendering glucan synthase insensitive to the drug (Satish *et al.* 2019).

In summary, for *A. fumigatus,* antifungal tolerance to echinocandins is associated with physiological changes in cell wall and membrane composition; tolerance to antifungal azoles remains to be investigated.

AZOLE RESISTANCE MECHANISMS

Azole resistance in *A. fumigatus* is mainly associated with the acquisition of mutations in and overexpression of *CYP51A*, and overexpression of efflux pumps, as discussed in detail below. Adaptation to a new environment before acquisition of stable mutations in the azole drug target, *e.g.*, *CYP51A*, requires orchestration of a rapid, robust, and coordinated response that allows the cell to thrive despite the presence of drugs. It has been shown that mounting an appropriate physiological response and phenotypic plasticity to itraconazole may take 60 min (Hokken *et al.* 2019). Among the most highly expressed genes 30 and 60 min after itraconazole exposure are efflux pump genes *mdr1* and *mdr4*; *hmg1*, encoding a 3-hydroxyl-3-methylglutaryl-CoA 1 reductase from the mevalonate pathway; and *ERG6*, the most upregulated gene at all time points studied, involved in ergosterol biosynthesis (Hokken *et al.* 2019) (Fig. 2). Further, increased expression of *hmg1* may lead to an increased production of mevalonate, a precursor in sterol biosynthesis, which can positively regulate the overexpression of *ERG6*, resulting in increased eburicol levels (Yasmin *et al.* 2012, Hokken *et al.* 2019). The higher activity of ERG6 also results in the higher quantity of eburicol, the substrate for CYP51A, which also results in higher expression of CYP51A to keep up with the substrate elevation. The detailed mechanisms of azole resistance are discussed below (Fig. 2).

Role of *CYP51A* and *CYP51B* in azole resistance

In *Candida* species, azole drugs target ERG11 catalyses demethylation of 14-α-lanosterol. In *A. fumigatus*, ERG6 catalyses this reaction and acts upstream of the triazole drug target cytochrome P450 51 (Fig. 2). Cytochrome P450 51 is encoded by two isoforms, *CYP51A* and *CYP51B* (Alcazar-Fuoli *et al.* 2008, Hokken *et al.* 2019), which share 59–63 % similarity (Warrilow *et al.* 2010, Hargrove *et al.* 2015). A gene essentiality study in *A. fumigatus* based on the gene expression levels revealed that *CYP51A* encodes the major enzyme required for mycelial growth; the biological function of *CYP51B* remains elusive (Hu *et al.* 2007). Further, deletion of *CYP51A* abrogates fluconazole resistance, but this effect is not observed when *CYP51B* is deleted (Mellado *et al.* 2005). In addition, *CYP51A* weakly binds to triazoles, which is in contrast with tighter binding observed for *CYP51B* (Warrilow *et al.* 2010). Collectively, these observations indicate that *CYP51A* is the major enzyme in the ergosterol biosynthesis pathway and azole resistance. The presence of both *CYP51A* and *CYP51B* is vital to cell survival, and deletion of either isoform is compensated by the presence of other, without major apparent abnormalities in cell morphology (Hu *et al.* 2007). Further, the intrinsic resistance of *Aspergillus* to fluconazole is thought to be mediated by I301T substitution in *CYP51A* and a higher expression of *CYP51A* than *CYP51B* upon fluconazole exposure (Blosser & Cramer 2012, Leonardelli *et al.* 2016).

Mutations in *CYP51A* and *CYP51B*

Mutations in the *CYP51A* gene identified in clinical and environmental ARAF isolates are listed in Tables 2 and 3. Most of these mutations within the *CYP51A* coding sequence are accompanied by tandem repeats (TRs) in the promoter region, such as TR_{34}/L98H or TR_{46}/Y121F/T289A (TR-mediated azole resistance mechanism is detailed in the following section) (Fig. 3). The effect of the mutations on MIC values and CYP51A structure requires high-resolution structure and/or simulation analysis. Although the crystal structure of CYP51B has been determined, that of CYP51A has not yet been defined (Hargrove *et al.* 2015). Therefore, the association of such mutations with azole resistance is mainly derived from heterologous expression experiments and simulation studies, in which CYP51A from human, *Mycobacterium tuberculosis*, and *Saccharomyces cerevisiae*, and CYP51B from *A. fumigatus* are used as models to evaluate the effect of specific amino acid substitutions on protein structure (Liu *et al.* 2016, Nash & Rhodes 2018). According to *in silico* modelling, the impact of an amino acid substitution on CYP51A structure depends on the position and the substituted residue (Liu *et al.* 2016). For instance, substitutions of G54, L98, M220, and Y431 decrease the binding affinity of CYP51A to azoles, while substitutions of G432 and also L98 reduce the stability of CYP51A, which can lead to conformational changes in the substrate and/or inhibitor binding pocket by causing dramatic changes in specific loop structures close to these sites (Liu *et al.* 2016, Nash & Rhodes 2018). Furthermore, L98H substitution reduces hydrogen bond formation between the residue at site 98 and polar side chains of adjacent residues, which could prevent docking of triazoles in the binding pocket (Nash & Rhodes 2018). Typically, L98 changes are accompanied by amino acid substitutions of S297 and F495, which are both adjacent to the binding pocket and, hence, may synergistically confer azole resistance (Liu *et al.* 2016). Conversely, G54, G138, and M220 are close to the opening channels 1 and 2, which are close to the ligand access channel. Based on the same analysis, substitutions of amino acids located on the periphery of the protein, such as E130D, L252L, S400I, F46Y, M172V, N248T, D255E, L358L, E427K, and C454C, do not cause pronounced conformational changes as they are far from the critical residues and, therefore, do not play a role in azole resistance (Liu *et al.* 2016).

Resistance mechanisms that do not involve *CYP51A* mutations

Upregulation of CYP51A

Upregulation of *CYP51A* expression is an important mechanism of azole resistance. It is partly mediated by a steroid regulatory element-binding protein (SREBP), SrbA. Apart from *CYP51A*, SrbA also controls many other genes involved in sterol biosynthesis, adaptation to hypoxic conditions, virulence, normal cell polarity and hyphal morphogenesis, iron uptake, nitrate assimilation, and heme biosynthesis (Willger *et al.* 2008, Chung *et al.* 2014, Dhingra & Cramer 2017). Following complex processing in the endoplasmic reticulum and Golgi apparatus, the N-terminal

DNA-binding domain of SrbA, a basic helix-loop-helix leucine zipper transcription factor, is liberated and translocated to the nucleus, where it binds to steroid regulatory elements (SRE) and activates the transcription of target genes (Fig. 3) (Willger *et al.* 2008, Chung *et al.* 2014, Dhingra & Cramer 2017). SRE elements typically contain two SrbA recognition sites, SRE1 and SRE2 (Willger *et al.* 2008, Gsaller *et al.* 2016). Hence, TRs identified in ARAF isolates (TR_{34}, TR_{48}, TR_{53}, and TR_{120}) act as additional SrbA-binding motifs, leading to the recruitment of additional SrbA molecules, and increased expression of *srbA* and *CYP51A*, and, to a lesser extent, *CYP51B* (Willger *et al.* 2008, Gsaller *et al.* 2016). Indeed, the activity of SrbA is similar to that of Upc2 in *C. albicans*, which upregulates the expression of *ERG11* (Willger *et al.* 2008, Gsaller *et al.* 2016). It should be noted that *CYP51A* is not exclusively regulated by SrbA, since *CYP51A* expression is not completely inhibited in mutants lacking *srbA* (Blosser & Cramer 2012). Of note, a 1822-bp insertion (type II transposon *Aft1*) was identified upstream of the start codon of *CYP51A* in an ARAF isolate overexpressing *CYP51A*; however, its role in azole resistance remains to be determined (Albarrag *et al.* 2011). Finally, although upregulation of *CYP51B* in ARAF isolates appears to be rare, a baseline and/or induced overexpression of *CYP51B* has been observed in a limited number of clinical isolates lacking mutations in *CYP51A* (Buied *et al.* 2013).

CCAAT-binding complex-mediated azole resistance

Whole-genome sequencing of an ARAF strain with wild-type (WT) *CYP51A* isolated from a Dutch patient identified HapE, a new factor involved in the regulation of sterol synthesis (Camps *et al.* 2012a). HapE is a subunit of the CCAAT-binding complex (CBC); it harboured the amino acid substitution P88L in the clinical isolate (Camps *et al.* 2012a).

CBC is a trimeric transcription factor complex (HapB, HapC, and HapE), which together with the monomeric transcription factor HapX regulates sterol synthesis by binding at positions −293 to −289 and −275 to −269 upstream of the *CYP51A* start codon, respectively (Fig. 3) (Gsaller *et al.* 2016). Knock-out analysis of CBC subunit genes and *hapX*, and heterologous expression of $HapE^{P88L}$ result in increased triazole MIC values and overexpression of CYP51A, HMG-CoA synthase (paralog of *erg13A* and *erg13B*), and HMG-CoA reductase (paralog of *hmg1*), indicating that these transcription factors act as repressors of genes involved in sterol biosynthesis (Gsaller *et al.* 2016). Further studies revealed that the N-terminal DNA-binding domains of CBC and HapX physically interact with one another (Hortschansky *et al.* 2015). Further, experiments under iron-limiting conditions demonstrated that the initial binding of CBC to a CBC motif allows the recruitment of HapX to HapX motif (5′-GAT-3′) located 11−12 bp downstream of the CBC motif (Hortschansky *et al.* 2015). Interestingly, although both SrbA and CBC competitively bind to the same SRE site, position −293, the binding affinity of SrbA is 8-fold higher than that of CBC (Gsaller *et al.* 2016). Further, SrbA shows a higher binding affinity for the original motif located in position −293 than to the additional motifs in isolates with TRs, located in position −327 (Gsaller *et al.* 2016). These studies implicated other determinants involved in azole resistance, which negatively regulate the expression of azole drug target and other genes involved in ergosterol biosynthesis.

The role of efflux pumps

Identification of a relatively large number of clinical ARAF isolates lacking *CYP51A* mutations and comparative genomics studies in yeasts prompted the investigation of alternative mechanisms of azole resistance. This led to the discovery of the role of efflux pumps in azole resistance. Efflux pumps are categorised into two main classes, the major-facilitator superfamily (MFS), encoded by 278 genes, and ATP-binding cassette (ABC) proteins that require ATP for activity, encoded by 49 genes (Loiko & Wagener 2017).

An initial comparative genomics analysis revealed that the two paralogs *CDR1A* and *CDR1B* (also known as *abcC*) are orthologous to *C. albicans CDR1*, and *abcA* the same as *Afumdr1* (Fraczek *et al.* 2013). The efflux pump genes *atrI* and *mdrA* partially contribute to azole resistance in clinical isolates, while *atrF* appeared to be upregulated in environmental ARAF isolates (Meneau *et al.* 2016). Studies focused on generating AFAR strains *in vitro* (UV-irradiated and itraconazole-treated strains) and those exploring the response of *A. fumigatus* to itraconazole and voriconazole revealed a pronounced overexpression of several efflux genes, namely, *Afumdr1*, *Afumdr3*, *Afumdr4*, and *atrF* (Nascimento *et al.* 2003, da Silva Ferreira *et al.* 2004, 2006b, Hokken *et al.* 2019). Of note, the triazole MIC values of isolates lacking *Afumdr1* and *mfs56* showed a slight change, which raises the question whether these proteins are the main efflux pumps involved in triazole resistance (Fraczek *et al.* 2013). Several studies dissecting the molecular mechanisms underpinning triazole resistance in clinical ARAF isolates, however, showed that *atrF*, *mfsC*, and *CDR1B* (orthologous to *CaCDR1*), especially *CDR1B* and *atrF*, are greatly upregulated in these isolates (Slaven *et al.* 2002, Fraczek *et al.* 2013, Meneau *et al.* 2016, Paul *et al.* 2017, Sharma *et al.* 2019, Wu *et al.* 2020). This observation is further supported by the observation that heterologous expression of *CDR1B* in *S. cerevisiae* isolate lacking *Pdr5*, or deleting this gene from an ARAF isolate carrying TR_{34}/L98H, TR_{34}, or L98H, results in a profound decrease of voriconazole MIC values (Paul & Moye-Rowley 2013, Paul *et al.* 2017). Furthermore, these efflux pumps have different substrate specificity, *i.e.*, a narrow or broad-spectrum and functionality, with CDR1B showing the broadest substrate specificity (Esquivel *et al.* 2020).

Attempts to identify the regulator of *CDR1B* expression resulted in the identification of AtrR, (ABC transporter−regulating transcription factor). AtrR is a Gal-4 type Zn_2-Cys_6 cluster-containing transcription factor, which shares homology with *CgPdr1* (Fig. 3) (Hagiwara *et al.* 2017). Interestingly, AtrR is responsible for the upregulation of *CYP51A* and also *CYP51B*, as predicted by experiments that suggested that proteins other than SrbA also control *CYP51A* expression (Blosser & Cramer 2012, Paul *et al.* 2019). Intriguingly, the AtrR response element (ATRE) is located within the 34-bp repeat element and, hence, both AtrR and SrbA share overlapping binding sites (Fig. 3) (Paul *et al.* 2019). Therefore, it is plausible that both SrbA and AtrR cooperatively mediate the upregulation of *CYP51A* expression, as well as the expression of several genes that control the ergosterol biosynthesis pathway (Hagiwara *et al.* 2017, Paul *et al.* 2019). Importantly, deletion of *atrR* resulted in azole hypersensitivity of a strain with CYP51A with G54E substitution, which indicates that AtrR contributes to azole resistance even in isolates with mutated *CYP51A* (Hagiwara *et al.* 2017).

Strains lacking both *atrR* and *srbA* are viable (Hagiwara *et al.* 2017), which may indicate the presence of other transcription factors that control the expression of other efflux pumps.

Although the regulator of *atrF* expression remains to be identified in *A. fumigatus*, a recent study using whole genome sequencing of a laboratory-derived voriconazole-resistant *A. flavus* isolate revealed that *yap1* controls the expression of *atrF* (Ukai *et al.* 2018). Yap1 is a bZIP master-regulator transcription factor involved in the oxidative stress response (Ukai *et al.* 2018). Interestingly, the voriconazole-resistant *A. flavus* isolates harboured the mutation Yap1^{L558T}, which resulted in *atrF* overexpression, and reverting Yap1^{L558T} to WT form and deletion of *atrF* significantly decreased the voriconazole MIC values (8- to 16-fold) (Ukai *et al.* 2018). The gain-of-function mutation resulted in changes in the C-terminus of Yap1, leading to a constitutive localisation of this transcription factor in the nucleus, binding to a putative Yap1 response element (YRE) at positions −462 to −456 relative to the start codon of the target genes, and overexpression of the target genes (Ukai *et al.* 2018). Of note, YRE is also present upstream of *CYP51A*, but this gene was not greatly upregulated in the respective azole-resistant isolate, which suggests that Yap1 does not control the CYP51A. This finding encourages evaluation of the *yap1* sequence in ARAF isolates overexpressing *atrF*.

Hmg1 and azole resistance

Losada *et al.* (2015) identified Hmg1 as another player in azole resistance, in an experiment involving successive *in vitro* exposure of *A. fumigatus* to various azole compounds. Hmg1 is bound to the endoplasmic reticulum membrane via an N-terminal anchor domain linked to the catalytic site via a linker (Fig. 3) (Sever *et al.* 2003, Friesen & Rodwell 2004). In the experiment of Losada *et al.* (2015), all voriconazole-resistant progenies had specific amino acid substitutions in the sterol-sensing domain of Hmg1. Sterol negatively regulates the activity of Hmg1, and in the presence of high sterol levels, the membrane-bound domain of Hmg1 is targeted to proteasome-mediated proteolysis (Sever *et al.* 2003). Therefore, it is plausible to associate mutations in the sterol-sensing domain with an increased enzyme stability, which would lead to sterol overproduction and, potentially, azole resistance (Sever *et al.* 2003, Friesen & Rodwell 2004, Losada *et al.* 2015, Jiang *et al.* 2018). Indeed, this was the case with ARAF clinical isolates from Japan and the US (Hagiwara *et al.* 2018, Rybak *et al.* 2019). These isolates, lacking *CYP51A* mutations but harbouring mutations in *hmg1*, produced more ergosterol and were more susceptible to polyenes, such as AMB, than isolates without mutations in *hmg1*. Other studies conducted in India and Taiwan identified similar mutations in *hmg1* in clinical ARAF isolates (Sharma *et al.* 2019, Wu *et al.* 2020). Intriguingly, a study conducted in the US revealed that a high proportion of isolates with WT *CYP51A* (11/21; 52 %) harbour mutations in the *hmg1* portion encoding the sterol-sensing domain (Siopi *et al.* 2017). Although initial ectopic expression experiments failed to associate the discovered *hmg1* mutations with azole resistance, a recent study using CRISPR-Cas9 methodology revealed that F262_del, S305P, and I412S dramatically increase the triazole MIC values (Rybak *et al.* 2019). Similarly, other genes involved in ergosterol biosynthesis, including *ERG6*, are mutated and may potentially contribute to triazole resistance, although these mutations are not as prevalent as those of *hmg1* (Hagiwara *et al.* 2018).

Master regulators of azole resistance

It is still unclear how genes involving ergosterol biosynthesis and SrbA, AtrR, CDR1B, and Hap complex genes are regulated on a larger scale, and which master regulators control their expression. In a recent study, potential master regulators that could simultaneously be involved in azole resistance and pathogenicity were systematically analysed (Furukawa *et al.* 2020). The authors showed that negative cofactor two (Nct2), consisting of the NctA and NctB subunits, regulates ergosterol biosynthesis and iron-responsive pathways by co-localising and interacting with the TATA-box located upstream of the target genes (an estimated nearly 30 % of coding genes in *A. fumigatus*). Interestingly, *nctA* and *nctB* mutants are not only pan-azole and AMB resistant, but they present no fitness cost as their pathogenicity is comparable with that of the WT (Furukawa *et al.* 2020). The controversial AMB resistance despite a modest increase of ergosterol content could be explained by an upregulation of oxidative stress-reducing enzymes and the notion that altered cell wall morphology may act as a barrier to AMB penetration in these mutants (Furukawa *et al.* 2020). These findings warrant future studies to assess the role of loss-of-function mutations in Nct2 complex genes in clinical ARAF isolates.

Additional mechanisms of azole resistance

In addition to the already mentioned major mechanisms of azole resistance, some other, relatively rare, mechanisms are also implicated in azole resistance. Damage resistance protein 1 (Dap1) is a cytochrome b_5-like heme-binding protein that regulates the function of CYP51A and ERG5. It is located at the endoplasmic reticulum membrane and is composed of three subunits, DapA, DapB, and DapC. DapA stabilises CYP51A and ERG5, allowing electron transfer, while DapB and DapC suppress electron transfer and prevent the activity of target proteins through depletion of heme (Song *et al.* 2016). Of note, although DAPA and DAPC co-localise at the endoplasmic reticulum membrane, and form complexes with CYP51A and ERG5, DAPB is located in the nucleus (Song *et al.* 2016, 2017). Gene deletion analysis revealed that *ΔdapA* was susceptible and *ΔdapC* was more resistant against itraconazole, while the itraconazole susceptibility of *ΔdapB* was indistinguishable from the parental strain (Song *et al.* 2016). The observation that even upon azole stress Dap1 family proteins remain at the endoplasmic reticulum membrane indicate that other transcription factors that translocate to the nucleus may regulate the expression of these genes (Song *et al.* 2017). In keeping with this anticipation, it was revealed that SrbA is required for the overexpression of *dapA* and *dapC*, and Dap1 family proteins *per se* do not sense ergosterol depletion, indicating that the expression of Dap1 protein genes is controlled by SRE (Song *et al.* 2017).

According to a recent study, mutation in a gene encoding farnesyl transferase (Afcox10^{R243Q}) and loss of *algA*, a component of the calcium signalling pathway, leads to itraconazole resistance (Wei *et al.* 2017). Interestingly, a collection of ARAF clinical isolates with WT *CYP51A* harbour several amino acid substitutions in Afcox10 but their effect on azole resistance was not evaluated (Sharma *et al.* 2019). A mismatch repair gene (*MMR*, also known as *MSH2*) plays an important role in facilitating the acquisition of drug resistance in *C. glabrata* (Healey *et al.* 2016). Unlike *C. glabrata*, however, the clinical and environmental *Aspergillus* isolates do not harbour many nonsense mutations in *MSH2.* Nonetheless, deleting *msh2* profoundly

Table 4. Studies using in-house and commercial methodologies for the direct detection of azole resistance in *A. fumigatus* from clinical samples.

Method	Type of assay	*CYP51A* mutations detected	Clinical samples (n)	*CYP51A* amplification (false positives)	Additional results	References
Conventional PCR + sequencing	In-house	TR_{34}, L98H, M220	BAL fluids (6); tissue (2)	100 %	75 % WT; 12.5 % TR_{34}/L98H, 12.5 % L98H	Spiess *et al.* 2012
	In-house	TR_{34}, L98H, M220, TR_{46}	Blood (25); BAL fluids (120); CSF (19); tissue (17)	100 % (39.8 %)	58.6 % WT; 1.1 % TR_{34}/L98H; 0.5 % L98H	Spiess *et al.* 2014
	In-house	TR_{34}, L98H, M220, TR_{46}, Y121F, T289A	BAL fluids (22)	74.2 % (65.1 %)	7.6 % not sequenced; 1.5 % TR_{34}/L98H	Postina *et al.* 2018
			Tissue (15)	68.9 % (54.5 %)	7.8 % not sequenced; 1.1 % L98H; 3.3 % TR_{46}/Y121F/T289A; 2.2 % TR_{34}/L98H	
			CSF (15)	39 % (28.7 %)	10.3 % not sequenced	
Pyrosequencing	In-house	G54	Blood (56)	3.6 %	3.6 % WT	Trama *et al.* 2005
	In-house	All of those described	Respiratory samples			van der Torre *et al.* 2020
Real-time PCR	In-house	TR_{34}, L98H	Sputum (1); tissue (4)	60 %	60 % TR_{34}/L98H	van der Linden *et al.* 2010
	AsperGenius®	TR_{34}, L98H, Y121F, T289A	BAL fluids (77)	18.2 %	15.6 % WT; 1.3 % TR_{34}/L98H; 1.3 % TR_{46}/Y121F/T289A	Chong *et al.* 2015
	AsperGenius®	TR_{34}, L98H, Y121F, T289A	Serum (72)	16.7 %	6.9 % TR_{46}/Y121F/T289A; 5.6 % L98H; 2.8 % TR_{34}/L98H; 1.4 % Y121F	White *et al.* 2015a
	AsperGenius®	TR_{34}, L98H, Y121F, T289A	BAL fluids (201)	33.8 %	28.3 % WT; 3.5 % TR_{34}/L98H; 1.5 % TR_{34}/L98H+WT; 0.5 % TR_{46}/Y121F/T289A	Chong *et al.* 2016
	MycoGENIE®	TR_{34}, L98H	Respiratory samples (88); serum (69)		0 % TR_{34}/L98H	Dannaoui *et al.* 2017
	AsperGenius®	TR_{34}, L98H, Y121F, T289A	BAL fluids (100)	20 %	17 % WT; 3 % unspecified mutations	Montesinos *et al.* 2017
	AsperGenius®	TR_{34}, L98H, Y121F, T289A	BAL fluids (91)	49.5 %	34.1 % WT; 8.8 % TR_{34}/L98H; 3.3 % TR_{34}/L98H +WT; 3.3 % TR_{46}/Y121F/T289A	Schauwvlieghe *et al.* 2017
	AsperGenius®	TR_{34}, L98H, Y121F, T289A	Plasma (86)		100 % WT	White *et al.* 2017
	AsperGenius®	TR_{34}, L98H, Y121F, T289A	Serum (9); tissue (8)	76.5 %	76.5 % WT	de Groot *et al.* 2018
	MycoGENIE®	TR_{34}, L98H	BAL fluids (31)		0 % TR_{34}/L98H	Denis *et al.* 2018
	AsperGenius®	TR_{34}, L98H, Y121F, T289A	Sputum (119)	47.9 %	47.9 % WT	Guegan *et al.* 2018
	MycoGENIE®	TR_{34}, L98H			0 % TR_{34}/L98H	Guegan *et al.* 2018
	MycoGENIE®	TR_{34}, L98H	Respiratory samples (147)		0 % TR_{34}/L98H	Burckhardt & Zimmermann 2018
	AsperGenius®	TR_{34}, L98H, Y121F, T289A	BAL fluids (22)	59.1 %	56.8 % WT; 2.3 % TR_{34}/L98H	Postina *et al.* 2018
			Tissue (15)	46.7 %	43.4 % WT; 3.3 % TR_{46}/Y121F/T289A	
			CSF (15)	41.7 %	41.7 % WT	
	MycoGENIE®	TR_{34}, L98H	BAL fluids (123)		0.8 % TR_{34}/L98H	Mikulska *et al.* 2019
	AsperGenius®	TR_{34}, L98H, Y121F, T289A	BAL fluids (23)	65.2 %	52.2 % WT; 8.7 % TR_{34}/L98H; 4.3 % TR_{46}/Y121F/T289A	Pelzer *et al.* 2020
Nested PCR + real-time PCR	In-house	TR_{34}, L98H, G54, G138, M220	Sputum (29)	100 %	48.3 % TR_{34}/L98H; 31 % L98H; 6.9 % TR_{34}; 6.9 % TR_{34}/L98H+M220; 6.9 % M220	Denning *et al.* 2011
	In-house	TR_{34}, L98H, G54, G138, M220, G448	BAL fluids (94)	64.9 %		Zhao *et al.* 2013
	In-house	TR_{34}, L98H, G54, G138, M220, G448	Respiratory samples (97)	39.2 %		Zhao *et al.* 2016
LAMP	In-house	TR_{34}	Clinical samples (11)		100 % TR_{34}/L98H	Yu *et al.* 2019

LAMP, loop-mediated isothermal amplification; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; WT, wild-type.

impacts genetic stability, antifungal resistance, and virulence in *A. fumigatus* (Dos Reis *et al.* 2019). Finally, some studies have implicated OrmA, the rate-limiting enzyme of the sphingolipid biosynthesis pathway (Zhai *et al.* 2019), b5 CybE (Misslinger *et al.* 2017), mitochondrial dynamics (Neubauer *et al.* 2015), and oxidoreductase HorA (Kroll *et al.* 2016), in azole resistance.

It should be noted that triazole resistance is a multifactorial phenomenon, involving alteration of the drug target, and upregulation of the drug target and efflux pumps (Nascimento *et al.* 2003, Fraczek *et al.* 2013). In *Candida* species belonging to the CTG clade, such as *C. albicans*, gain-of-function mutations in *UPC2*, *TAC1*, and *MRR1* result in upregulation of *ERG11* and efflux pump genes (Gsaller *et al.* 2016). Since genes with similar function exist in *A. fumigatus*, *e.g.*, *srbA* and *atrR*, it would be interesting to explore the presence of such gain-of-function mutations in ARAF isolates, and their association with *CYP51A* and *CDR1B* overexpression. Further, gain-of-function mutations in *CgPDR1* lead to increased virulence and immune evasion and, hence, their implications for *A. fumigatus* represent an interesting area for future investigations (Vale-Silva *et al.* 2013). The most important factor that would facilitate gene expression analysis in *A. fumigatus* is gene characterisation, since most genes remain uncharacterised, which makes the interpretation of transcriptomic studies challenging (Hokken *et al.* 2019). Nonetheless, the current understanding of azole resistance in *A. fumigatus* offers a wide range of potential targets that can inspire the development of novel and potent antifungal drugs.

Azole resistance and in-host fitness cost of *A. fumigatus*

Aspergillus fumigatus exhibits tremendous phenotypic, physiologic, and genomic plasticity, which allows it to adapt to and survive azole exposure. However, *bona fide* azole resistance requires the acquisition of permanent mutations in azole resistance-conferring genes and/or rewiring of the transcriptomic landscape to enable fungal persistence and survival in the presence of antifungal drugs within the host. Occurrence of such changes, in turn, results in therapeutic failure and dramatically increases the mortality rates, to over 80 % and even up to 100 % in real-life clinical settings (van der Linden *et al.* 2011, van Paassen *et al.* 2016). However, since resistance-conferring mutations negatively affect the catalytic activity of key enzymes, such as CYP51A, the mutated isolates may show fitness defects in the absence of azoles compared with the WT population. Indeed, the growth rate and conidia production by isolates harbouring various mutations and with large chromosomal deletions are markedly lower than those of isogenic susceptible isolates (Hagiwara *et al.* 2014). Further, as shown in *in vivo* studies, ARAF isolates harbouring HapE with P88L are less virulent than azole-susceptible isogenic ancestors and WT isolates, and exhibit a 4-h growth delay relative to susceptible and WT isolates (Arendrup *et al.* 2010). In keeping with these observations, ARAF isolates are not detected following discontinuation of the azole therapy, while resistant isolates reappear following itraconazole treatment in the clinical setting (Chen *et al.* 2005). By contrast, ARAF isolates carrying mutations in *CYP51A*, especially those with TRs, may harbour additional mutations in the genome, acting as a compensatory mechanism, which allow them to thrive within the host and/or the environment (Verweij *et al.* 2016b). Indeed, no significant differences in the sterol (and ergosterol) content of several of azole susceptible and ARAF isolates were detected in one study (Alcazar-Fuoli *et al.* 2008). These observations highlight the notion that while mutations might affect the docking of an antifungal at the enzyme active site, they might not affect the binding of the sterol substrate, as also suggested in simulation studies (Nash & Rhodes 2018). Further, the presence of two copies of the *CYP51* gene might enable rapid ergosterol biosynthesis and increased azole resistance (Hu *et al.* 2007). The presence of multiple paralogs impacts the fitness cost, which is unexpected, based on what is known about yeasts species, such as *Candida*. Consistent with this notion, in one study, mutant ARAF isolates were persistently isolated from clinical samples of a patient following discontinuation of azoles (Tashiro *et al.* 2012), and isolates carrying TR_{46}/Y121F/T289A exhibit the same growth rate and conidia production on PDA medium as WT isolates (Hagiwara *et al.* 2016).

There are multiple possible explanations for such contradictory observations. First, since the fitness cost is an outcome of accumulation of multiple mutations in the genome (Verweij *et al.* 2016b), assessment of the effect of various mutations requires a study of isogenic isolates, which only differ with respect to the presence of mutations of interest in a locus of interest. Accordingly, WT status should not be assigned solely on the sequencing of *CYP51A* or few genes. Indeed, to obtain reliable data, studies focusing on fitness-cost evaluation should utilise a well-defined WT isolate whose entire genome has been sequenced. Second, the fitness cost may vary depending on the gene and mutation studied, *e.g.*, the virulence attenuation observed in $HapE^{P88L}$ strains (Arendrup *et al.* 2010) *vs.* lack of pronounced virulence attenuation in isolates lacking *nct2* (Furukawa *et al.* 2020). Third, pronounced growth defects *in vitro* do not always mirror isolate behaviour *in vivo*, since, as shown in some studies, the *in vivo* virulence of mutant strains with a growth defect *in vitro* does not significantly differ from that of WT isolates (Furukawa *et al.* 2020), which reflects the complex nature of growth in the host. Further complicating matters is simultaneous recovery of both, azole-susceptible and -resistant isolates from clinical samples, which undermines the notion of predominance of a single genotype in a specific ecological niche. Indeed, a remarkable 20 % rate of isolation of mixed susceptible-resistant colonies from clinical samples was reported in one study (Fuhren *et al.* 2015), which reinforced the analysis of multiple isolates per clinical sample to verify the concomitant presence of isolates with different susceptibility profiles (Camps *et al.* 2012c).

Resistance to other classes of antifungals

As explained above, the continuous increase in the prevalence of triazole-resistant *A. fumigatus* in the clinic promote physicians to use other antifungal drugs, most notably AMB and echinocandins. Although the degree of resistance may vary depending on the fungicidal and fungistatic nature of these antifungals, as is the case with azoles, it is rational to assume that the selective pressure exerted by these antifungals will allow the selection of drug-resistant *A. fumigatus* isolates. Overall, the AMB resistance is a rare phenomenon among patients with IPA and although not generalising the case, some studies have shown that AMB MIC did not differ among IPA patients with/ without AMB exposure (Moosa *et al.* 2002). The AMB resistance rarity also may explain the lack of knowledge on underlying resistance mechanisms

Table 5. Advantages and disadvantages of technologies used for typing clinical and environmental *A. fumigatus* isolates.

Assay	Methodology	D*	R**	References
RAPD	Random amplified polymorphic DNA	Low	Low	Bertout *et al.* 2001
SSDP	PCR typing method combining RAPD and specific DNA primers	Low	Low	Mondon *et al.* 1997
MLEE	Multilocus enzyme electrophoresis	Low	Low	Rodriguez *et al.* 1996, Bertout *et al.* 2000
AFLP	Amplified fragment length polymorphism analysis	Moderate	Low	Warris *et al.* 2003
RFLP	Restriction fragment length polymorphism analysis	Low	Low	Neuveglise *et al.* 1996
MLP	Microsatellite length polymorphism	High	Moderate	Bart-Delabesse *et al.* 1998, Bertout *et al.* 2001
RISC	Retrotransposon insertion-site PCR amplification	Moderate	Moderate	de Ruiter *et al.* 2007
MLST	Multilocus sequence typing	Moderate	High	Bain *et al.* 2007
STR*Af*	Short coding tandem repeats	High	Moderate	de Valk *et al.* 2005, Guinea *et al.* 2011, Escribano *et al.* 2015, Fan *et al.* 2020
CSP	Cell-surface protein sequencing	Moderate	High	Balajee *et al.* 2007b, Levdansky *et al.* 2007
TRESPERG	Sequencing of tandem repeats surface protein coding genes	High	High	Garcia-Rubio *et al.* 2018a, Fan *et al.* 2020
WGS	Whole Genome Sequencing	High	High	Hagiwara *et al.* 2014, Garcia-Rubio *et al.* 2018b, Puértolas-Balint *et al.* 2019

D*, discriminatory power; R** reproducibility considering stability and availability.

involved in *A. fumigatus*, which appears to be associated with less drug uptake and a higher catalase activity in *A. terreus* (Blum *et al.* 2013). Although counterintuitive, the ergosterol level does not seem to differ among AMB-resistant and AMB-susceptible *A. terreus* isolates (Blum *et al.* 2013). To close this knowledge gap, developing *in vitro* AMB-resistant *A. fumigatus* isolates followed by unravelling the subcellular mechanism may provide some insights on this context.

Similar to AMB, echinocandin resistance is rarely reported among clinical isolates of *A. fumigatus* (Arastehfar *et al.* 2020b) and few studies conducted in this regard have shown that acquisition of mutations in a hotspot of the *FKS* gene, which encodes the catalytic subunit of β-1,3-D-glucan synthase (Jiménez-Ortigosa *et al.* 2017), along with the changes in the lipid profile surrounding β-1,3-D-glucan synthase (Satish *et al.* 2019) may serve as the possible cellular factors underlying echinocandin resistance.

ANTIFUNGAL DRUG RESISTANCE DETECTION IN *A. FUMIGATUS*: FROM PHENOTYPIC ASSAYS TO MALDI-TOF MS

As discussed, mortality rates are notably high in patients infected with *A. fumigatus* azole resistant strains (van der Linden *et al.* 2011, Lestrade *et al.* 2019), evincing the importance of making an early resistance detection in order to start an appropriate therapy. Antifungal susceptibility testing has the ultimate goal of helping clinicians to anticipate the chance of treatment success or failure. This generally follows the "90/60" rule in which approximately 90 % of the infections caused by susceptible isolates and 60 % of those due to resistant isolates respond to therapy (Rex & Pfaller 2002). Antifungal resistance can be *in vitro* detected by performing broth microdilution assays such as those developed and standardised by the Clinical and Laboratory Standard Institute (CLSI) (CLSI 2008) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Arendrup *et al.* 2017a), considered as the reference for yeasts and moulds. These methods determined the MIC, defined as the lowest drug concentration required to inhibit fungal growth. However, some antifungals are only able to partially inhibit certain species, causing changes in their hyphal growth. Such is the case of echinocandins on moulds, for which the two methodologies propose the determination of the minimum effective concentration (MEC), the lowest concentration of antifungal resulting in hyphal morphological changes (Kurtz *et al.* 1994). MEC assessment has the difficulty of being subjective and requiring expertise in microscopic observation in order to determine what is considered as aberrant growth. Even though there are some methodological differences between CLSI and EUCAST procedures, their results have been proved to be comparable (Espinel-Ingroff *et al.* 2013) and allow the categorisation of the strains as susceptible or resistant by applying the established clinical breakpoints (CBPs). Although EUCAST has defined drug-related and species-related CBPs for *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger* and *A. terreus* (EUCAST 2020), CLSI has recently adopted CBPs for voriconazole only for *A. fumigatus* species and uses epidemiological cut-offs (ECVs) to discriminate between wild-type susceptible strains and others with acquired resistance to other antifungal drugs (https://clsi.org/media/3682/m61ed2_sample.pdf, Espinel-Ingroff & Turnidge 2016).

In addition, several complementary methods have been commercialised and are easily carried out in the daily routine of a clinical microbiology laboratory, which include colorimetric endpoint methodologies such as Sensititre YeastOne, Micronaut-AM and the XTT assay, or agar-based methods using strips with a gradient of antifungal concentrations (Etest or MIC Test Strips) or four-well plates for azole (VIPCheck) and/or echinocandin susceptibility testing. The performance of these methods has been compared with the reference standard with generally positive results. Sensititre YeastOne yielded high essential agreement rates with CLSI for itraconazole, voriconazole, posaconazole and amphotericin B (Castro *et al.* 2004, Guinea *et al.* 2006, Mello *et al.* 2017), although its concordance for echinocandins was lower so its use is not recommended in this case (Siopi *et al.* 2017). Micronaut-AM showed good categorical agreements ($\geq$96 %) with CLSI for anidulafungin, amphotericin B, voriconazole and itraconazole, being able to detect azole

Table 6. Comparison of the main features of the three most widely used high-throughput sequencing platforms. Data has been compiled from vendors information and the literature mentioned in the text.

Platform	Advantages	Disadvantages	Comments
Illumina	– Ultra-high throughput – Low cost per sequencing coverage – Low error rate (<0.1 %) – Benchtop versions available (*i.e.* MiSeq, NextSeq).	– Short read length (< 300 bp)	– The high throughput and low error rate makes it ideal for re-sequencing projects when a reference is available. – Optimal for epidemiological studies where focus is on sequence variation of many samples. – Not suitable to analyze highly repetitive regions
Oxford Nanopore	– Ultra-long reads (median 20–50 kb, reaching up to 100 kb or more). – Simple and small instrumentation, portable.	– Medium throughput – High error rates in raw sequences (5–40 %)	– Read length enables easy assembly and taxonomic assignment of single reads. – Needs correction (updated versions are more accurate), often in combination with Illumina. – Cheap and portable equipment opens possibilities for clinical use.
PacBio	– Ultra-long reads (median (8–10 kb, up to 60 kb or more)	– Medium throughput – High error rates in raw sequences (10–15 %) – Large equipment	– Similar as above, but less amenable for in site operation, given the size and complexity of the equipment.

resistance in *A. fumigatus* (Nuh *et al.* 2020). As echinocandin susceptibility testing for *Aspergillus* spp. using reference methods is not easy, the XTT-based assay proved to be a feasible alternative and showed promising results when compared with EUCAST MEC values (Meletiadis *et al.* 2020). Etest and MIC Test strips have been confirmed to be reliable alternatives for antifungal susceptibility testing for *Aspergillus* species, as well as for detecting *A. fumigatus* azole resistance, after obtaining categorical and essential agreements of $\geq$90 % when correlating their results with those yielded by CLSI (Guinea *et al.* 2008, Martos *et al.* 2010, Lamoth & Alexander 2015) and EUCAST (Arendrup *et al.* 2017b, Idelevich *et al.* 2018). Azole and echinocandin resistance in *A. fumigatus* is successfully detected using four-well agar plates, showing comparable results to those obtained with the reference methodologies (Arendrup *et al.* 2017c, Buil *et al.* 2017b, Tsitsopoulou *et al.* 2018, Meletiadis *et al.* 2019). EUCAST has proposed several recommendations for their use as a screening procedure for the detection of azole resistance (Guinea *et al.* 2019).

Nevertheless, these methodologies have important limitations, especially for moulds: while EUCAST and CLSI are time-consuming and laborious, colorimetric and agar-based methods are easier to perform. Besides, all of them require a fungal pure culture. This can be a critical issue due to the usual low recovery rate of *Aspergillus* species in culture from clinical samples, which also leads to the underestimation of azole resistance rates (van der Linden *et al.* 2016). Although the potential of culturing high volume sputum samples yielded positive results for patients with chronic and pulmonary aspergillosis (Vergidis *et al.* 2020), the development of alternative non-culture-based techniques is essential.

In this context, molecular tools detecting resistance mutations directly from clinical samples are proving to be complementary to phenotypic assays by reducing turnaround times for the initiation of an effective therapy (Jenks *et al.* 2019f). Several in-house and commercial PCR-based methodologies to directly detect mutations in the *A. fumigatus* azole-resistance related gene *CYP51A* and its promoter, including the most frequent point mutations (G54, G138, M220, G448, L98H, Y121F, T289A) and tandem repeat insertions (TR_{34} and TR_{46}), have been developed (Table 4). While some consist in conventional PCR assays (Spiess *et al.* 2012, 2014, Postina *et al.* 2018), the vast majority are real-time PCR based methods that avoid the delay associated with sequencing, something also successfully achieved with loop-mediated isothermal amplification (LAMP) assays (van der Linden *et al.* 2010, Denning *et al.* 2011, Zhao *et al.* 2013, 2016, Chong *et al.* 2015, 2016, White *et al.* 2015a, 2017, Dannaoui *et al.* 2017, Montesinos *et al.* 2017, Schauwvlieghe *et al.* 2017, de Groot *et al.* 2018, Denis *et al.* 2018, Guegan *et al.* 2018, Morio *et al.* 2018, Mikulska *et al.* 2019, Yu *et al.* 2019, Pelzer *et al.* 2020). Pyrosequencing also shows promising results, as it has the advantage of being adaptable to other genes of interest (Trama *et al.* 2005, van der Torre *et al.* 2020). However, one of their main limitations is that they need to be very sensitive and specific to detect the low concentration of *Aspergillus* DNA and prevent cross-reactivity with human DNA. Besides, *CYP51A* is a single-copy gene, which impairs its amplification, although this can be improved by the use of a nested PCR assay in order to achieve a higher sensitivity (Denning *et al.* 2011, Zhao *et al.* 2013, 2016). Although other in-house and commercial molecular methods for detecting *CYP51A* mutations in *A. fumigatus* have been successfully developed, they have not been evaluated in clinical samples yet (García-Effron *et al.* 2008, Klaassen *et al.* 2010, Bernal-Martínez *et al.* 2017, Wang *et al.* 2019, Fungiplex® 2020). The emergence of CYP51A-WT strains increasingly being identified in clinical settings in addition to a diverse range of mutations occurring in the CYP51A challenge the applicability of such tools. Despite the limited nature of resistance-associated mutations, there may be a role for whole-genome sequencing as an alternative strategy (see section 13).

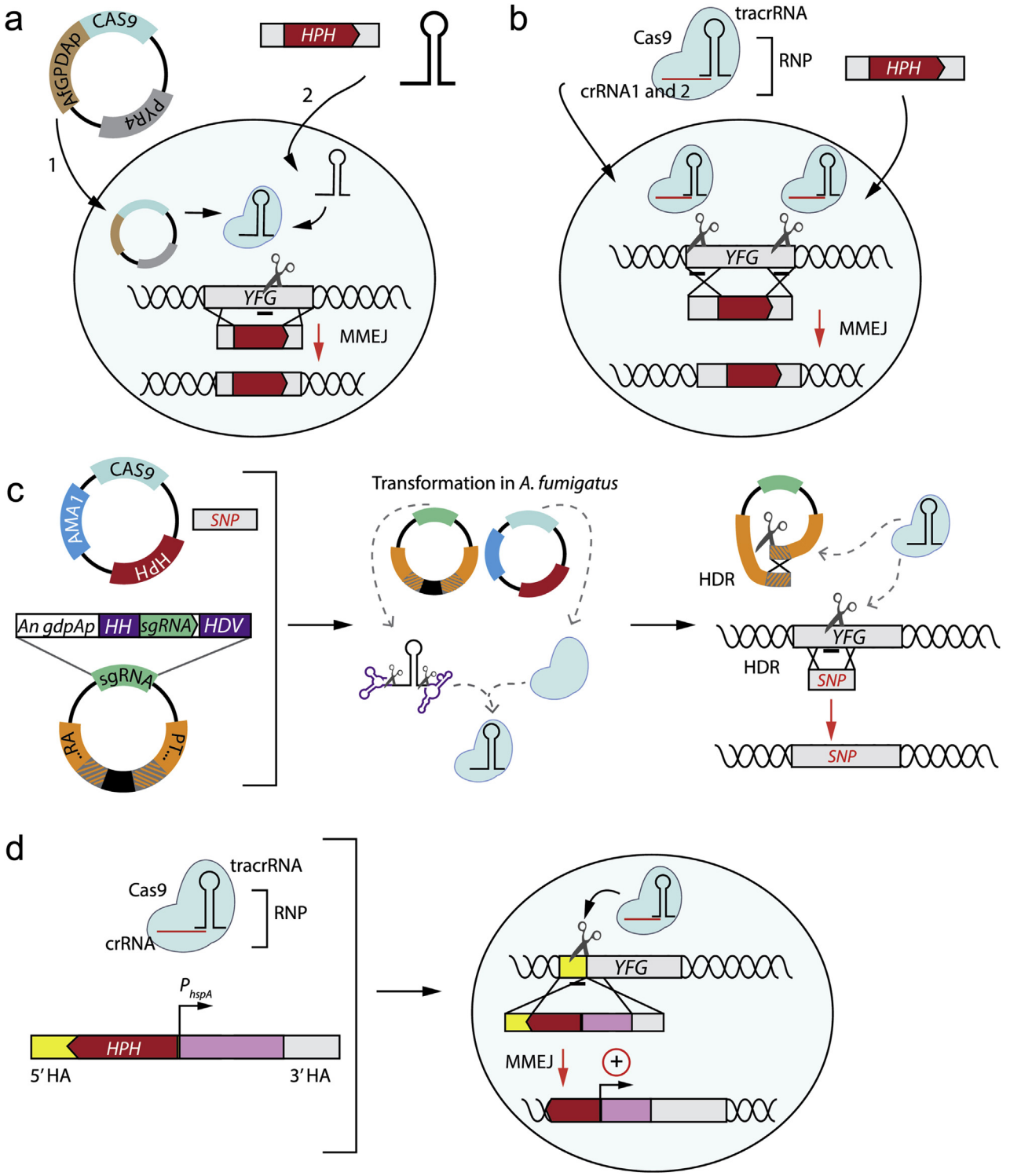


Fig. 4. CRISPR-Cas9 technology in *A. fumigatus*. The vignettes illustrate representative methods for CRISPR–Cas9 genetic manipulation of *A. fumigatus*. (a) MMEJ can be used to integrate an *HPH* cassette into a desired locus by using short homology arms. The strain is first transformed with a plasmid expressing Cas9 and containing a *PYR4* marker and then with an *in vitro* transcribed sgRNA and the *HPH* cassette. (b) The need to set up a suitable system to express the CRISPR elements can be circumnavigated by using CRISPR RNPs, in which two different crRNAs and tracrRNA are assembled *in vitro* with Cas9 and then transformed into the cells to target the upstream and downstream regions of *YFG*. MMEJ results in the integration of a *HPH* cassette into the targeted gene. (c) Ballard and colleagues tweaked the systems developed by into a two-plasmid system for introducing SNPs into a clinical isolate without marker integration. The *AMA1* sequence supports the replication of the plasmid harbouring *CAS9* in *A. fumigatus*, which confers resistance to hygromycin. A different plasmid carries a cassette for the expression of a ribozyme-flanked sgRNA from a *A. nidulans* RNA pol II promoter; after expression in *A. fumigatus*, the self-splicing activity of the rybozymes releases the mature sgRNA. This plasmid contains a *PTRA* split marker, interrupted by the same protospacer sequence that is being targeted on the gene of interest. After transformation of both plasmids and a RT containing the SNP to introduce in *YFG*, Cas9 targets both the protospacer on the desired locus in the genome and the twin protospacer interrupting the split marker. HDR then simultaneously mediates the insertion of the SNP into YFG and the reconstitution of the PTRA marker, thus allowing for selection of the transformants on pyrithiamine without marker integration. (d) The RNP system can also be exploited to affect gene expression, and it was recently used to replace a native promoter with a constitutive *hspA* promoter by transforming the cells with the RNP particle and a repair template carrying *HPH*, *hspA*, and homology arms flanking the insertion site. Af, *A. fumigatus*; CRISPR, clustered regularly interspaced short palindromic repeats; crRNA, CRISPR-RNA; HDR, homology-directed repair; HDV, human hepatitis delta virus; HH, hammerhead; MMEJ, microhomology-mediated end joining; RNA–Cas9 protein complex; sgRNA, single- guide RNA; tracrRNA, trans-activating RNA; YFG, your favourite gene. HA, Homology Arms; RT, Repair Template. Panels a and b were adopted from Morio *et al.* (2020) with permission.

Taking all of these into account, new molecular assays should intend to cover a broader range of azole resistance-related mutations and mechanisms, as not all of the *CYP51A* reported alterations are detected by the available methodologies, while increasing their sensitivity in order to become a feasible option to detect azole resistance in more clinical settings. Besides, there are no molecular options for the direct detection of azole resistance in *Aspergillus* species other than *A. fumigatus*, which should be further studied and developed.

Since MALDI-TOF MS has been introduced as a routine identification tool, laboratories are interested to explore this rapid technology as an alternative methodology potentially accelerating antifungal susceptibility testing (Burckhardt & Zimmermann 2018). A simple approach using specific marker peaks in mass spectra of resistant microorganisms could be applied to a number of specific resistances in certain bacteria, but this approach was yet not successful for fungi. Several functional assays have been proposed for antibiotic susceptibility testing in bacteria, *e.g.,* a test for the hydrolytic degradation of β-lactam antibiotics, the observation of incorporation of stable isotope-labelled metabolites, or the utilisation of MALDI-TOF MS as semi-quantitative read out for microbial growth in the presence of an antibiotic drug. Less work has been performed until today to apply such MALDI-TOF MS based assays to antifungal resistance. Most of the studies in this regard have focused on the detection of resistance in yeasts by MALDI-TOF MS. Detection of peak pattern changes after incubation of cells in presence of an antifungal has been demonstrated to determine antifungal resistance in *Candida* species (Delavy *et al.* 2019). In two studies, this method was also applied to test susceptibility in *Aspergillus* species. One study described the detection of caspofungin resistance in *A. fumigatus* and *A. flavus* strains (De Carolis *et al.* 2012). The same approach was applied to strains of *Aspergillus* species and voriconazole (Gitman *et al.* 2017). Although the results were in good agreement with reference methods, there was no obvious advantage over traditional methods, in particular because of the similar time to result. A promising method which has been shown to detect antifungal resistance in *Candida* by semi-quantitative detection of fungal growth in the presence of the drug after only several hours of incubation (Vatanshenassan *et al.* 2018, 2019) has not yet been applied to *Aspergillus* yet, according studies should be performed.

APPLICATION OF TYPING TECHNIQUES TO IDENTIFY INFECTION AND RESISTANCE ROUTES

Strain genotyping is considered one of the most basic tools in the clinical setting since it fulfils many needs, among which the establishment of epidemiological relationships between isolates stands out. As in many other settings, typing methodologies have had an important impact in the aspergillosis field, since they have been used, among many others, for outbreak analysis (Menotti *et al.* 2005, Doll *et al.* 2017), environmental monitoring of the isolates that constitute a specific population (Deng *et al.* 2017, Fan *et al.* 2020), also for patient monitoring in order to study how clinical strains evolve under drug pressure within the antifungal therapy (Escribano *et al.* 2017) or to assess local and global *Aspergillus* spp. epidemiology (Garcia-Rubio *et al.* 2018a, 2018c, Choi *et al.* 2019). Thus, the molecular analysis of the genetic and epidemiological relationship between environmental and clinical strains could potentially assess strain origin and route of transmission. Besides all these applications at the subspecies level, molecular typing methods have also been used at the genus level for discriminating between species and also for the definition and recognition of new fungal species (Klaassen & Osherov 2007).

Different methodologies have been developed to genotype *Aspergillus* species strains (Table 5). However, due to its clinical significance, most of them have been implemented for *A. fumigatus* strains (Latgé & Chamilos 2019). Classically, genotyping techniques can be grouped in two different categories; methods either based on PCR amplification and sequencing, which are described in detail below, or based on non-coding repetitive sequences paired with restriction fragment length polymorphisms, such as random amplified polymorphic DNA (RAPD) (Loudon *et al.* 1993) amplified fragment length polymorphism analysis (AFLP) (Warris *et al.* 2003), and restriction fragment length polymorphism analysis (RFLP) (Neuveglise *et al.* 1996). The latter ones show a poor inter-laboratory reproducibility which is the reason why they have been replaced by other techniques. Thus, the selection of the most appropriate method in each context will highly depend on the technical resources of a particular setting (Klaassen & Osherov 2007).

Although not a *bona fide* gold standard technique, the microsatellite analysis assay called STR*Af* is the most popular and widely used technique used to type *A. fumigatus*, which stands for short tandem repeats of *A. fumigatus* (de Valk *et al.* 2005). This assay, developed more than a decade ago, is based on a panel of microsatellites divided into three multicolour multiplex PCRs. Each multiplex reaction amplifies three di-, tri-, or tetranucleotide repeats, respectively. One of the biggest advantages of this technique is the multicolour multiplex approach which allows large numbers of markers to be tested in a short period of time, which is why this assay is a very suitable tool for large-scale epidemiological studies. Moreover, it can even be used to genotype *A. fumigatus* isolates directly from clinical samples, such as formalin-fixed paraffin-embedded tissues or serum samples (de Groot *et al.* 2018). The Simpson's diversity index of this assay (Hunter & Gaston 1988), which shows the discriminatory power of the methodology, is really high –0.9994 (de Valk *et al.* 2005), 0.988–0.995 (Escribano *et al.* 2015), 0.984 (Guinea *et al.* 2011) and 0.9993 (Garcia-Rubio *et al.* 2018a, 2018c). From a methodological point of view, the STR*Af* assay presents some major difficulties associated with sizing of the obtained PCR products; high-resolution equipment such as capillary-based or acrylamide-based electrophoresis platforms is required to translate the fragment electrophoretic mobility to their repeat number. However, this mobility is dependent on many critical factors such as the presence, or not, of denaturing compounds, the matrix, the run temperature, the sequence of the fragment, the fluorescent labels, the sizing marker, *etc.* (Klaassen & Osherov 2007). In order to get exchangeable typing results between laboratories, it is necessary to run allelic ladders for calibrating every platform (de Valk *et al.* 2009). Also, the low-

level instability of two of the markers (de Groot & Meis 2019), together with the availability of the required laboratory technology, dedicated software and personnel specifically trained for its performance comprise some of the main disadvantages of this assay (Klaassen & Osherov 2007, Garcia-Rubio *et al.* 2016).

As a result, the development of novel and more accessible typing methods has been encouraged. One simple and rapid single-locus typing method was developed based on sequencing the coding tandem repeats present on the cell surface protein (CSP) gene (Balajee *et al.* 2007b, Levdansky *et al.* 2007). This method has a lower discriminatory power than microsatellite-based typing – 0.78 (Klaassen *et al.* 2009), 0.83 (Gao *et al.* 2013) – and that is why a new typing method, called TRESPERG assay, was described combining four coding tandem repeat markers (Garcia-Rubio *et al.* 2018a, 2018c). This assay has a sufficiently high discriminatory power to compete with STR*Af*, and its main advantage is that it does not require trained personnel, specific equipment, or software for analysis, as it only consists of conventional PCR amplification and Sanger sequencing (Garcia-Rubio *et al.* 2018a, 2018c). Moreover, TRESPERG assay clustered tandem-repeat (TR) azole resistant strains in a better manner than STR*Af* assay did compared to whole genome sequencing (WGS) studies. Many authors have described these TR azole resistant strains as genetically more closely related than other *A. fumigatus* isolates (Camps *et al.* 2012b, Abdolrasouli *et al.* 2015, Garcia-Rubio *et al.* 2018b, Wang *et al.* 2018b). This fact is supported by TRESPERG results in which every *A. fumigatus* TR isolate tested grouped in only one cluster, endorsing their genetic closeness (Garcia-Rubio *et al.* 2018a, 2018c).

Finally, whole genome sequencing (WGS) has recently emerged as an invaluable tool for the analysis of genetic differences between *A. fumigatus* strains and has turned into the typing technique with the highest resolution, becoming increasingly affordable and widely available (Hagiwara *et al.* 2014, Garcia-Rubio *et al.* 2018b, Puértolas-Balint *et al.* 2019). The details of this technology is presented below.

WHOLE-GENOME SEQUENCING APPLICATIONS IN THE CLINIC

The phenotypic traits of an *Aspergillus* strain, including its virulence potential and its ability to survive drug exposure, are ultimately encoded in its genome. Reference genome sequences for *A. fumigatus*, and *A. nidulans* are available since 2005 (Galagan *et al.* 2005, Nierman *et al.* 2005), and have undoubtedly served to sustain major advances in the field. However, as many other fungi, *Aspergillus* have very plastic genomes and phenotypes, with clinically relevant traits varying widely across isolates. The lowering costs and continuing developments in high-throughput sequencing have recently allowed zooming into the specific genomes of particular strains, revealing how genomic and phenotypic plasticity are connected. In addition, our ability to access full genomic sequences in a timely and cost-effective manner are opening new avenues for clinical applications such as species identification and diagnosis of resistance potential (Consortium & Gabaldón 2019). Currently, three major sequencing platforms exist that differ in their functionalities and suitability for different purposes (Table 6). Their combined use has enabled deciphering genome variation in the *Aspergillus* genus with increasing level of resolution. For instance, reference genomes for virtually all major species in the genus have been produced in the last few years (Kjærbølling *et al.* 2018, 2020, Vesth *et al.* 2018). More recently, re-sequencing of evolved isolates has served to estimate mutation rates in the three major species (Álvarez-Escribano *et al.* 2019) – *A. flavus* (4.2×10^{-11} mutations per site and mitotic division), *A. fumigatus* ($1.1. \times 10^{-11}$) and *A. nidulans* (4.1×10^{-11}), which can be used, for instance, to more precisely date the divergence between two strains or the origin of clinical outbreaks. Other sequencing studies have shown that relationships between genome and phenotypic plasticity can be mapped by comparing genomes and phenotypes from different isolates (Bastos *et al.* 2020, Drott *et al.* 2020). The sequencing of serially collected isolates from the same patients with aspergillosis can reveal mutations acquired during the infection process (Hagiwara *et al.* 2014) and whole genome sequencing can identify mutations leading to azole resistance, particularly in resistant isolates not bearing mutations in *CYP51A* (Ukai *et al.* 2018, Sharma *et al.* 2019). Finally, another promising application of high-throughput sequencing is the direct detection of pathogenic species through targeted barcode sequencing or whole genome shotgun sequencing of complex samples, without the need of isolation, allowed by the high sensitivity of high-throughput sequencing approaches coupled to the increasing resolution power of comparisons with sequence databases (McTaggart *et al.* 2019).

Although these developments are very promising, the implementation of whole genome sequencing in the clinic faces important challenges which limits its implementation (Greninger 2018, Consortium & Gabaldón 2019, Kidd *et al.* 2019). Three major clinical applications of genome sequencing are considered: (i) genome-wide profiling of resistance conferring mutations, (ii) genome-wide molecular epidemiology for the study of outbreaks and, (iii) detection and identification of pathogens from complex, patient samples. For the three applications, analytical methodologies are ready, as we have seen in the examples above. However, the translation of these methodologies from an academic study to a clinical context faces many issues, of which we will discuss the more relevant. The first issue is cost. Although the prices of high-throughput sequencing equipment continue to drop, they are still far away from those of other routine analyses in the clinical mycology lab, particularly when one considers the combined costs of reagents, equipment purchase and maintenance, computational infrastructure, and necessary personnel (Greninger 2018). Another important aspect is time, particularly for diagnostic purposes, less so for epidemiological studies. Most sequencing approaches require multiple steps before sequencing can be run (isolation, culture, DNA extraction, library preparation, *etc.*). These factors coupled with the required bioinformatics analyses that are often not fully automatised, require high-level expertise, which delay results over the limits that are reasonable in the context of clinical needs. This problem can be aggravated if the sequencing and bioinformatics resources are not on site, which still is currently the case for most hospitals. Finally, despite many developments in computational tools and databases, they are still not mature for routine infection control and outbreak investigations. For instance, many public genome repositories are not curated, leading to wrong annotations that can lead to errors in species identification (Stavrou *et al.* 2018). In our view, full integration of such methodologies into the clinics requires the following

Table 7. Examples of mammalian models for different types of disease caused by *Aspergillus*.

Type of disease	Host species	Route of infection or exposure	Immunosuppression	References[1]
Invasive aspergillosis, pulmonary	Mouse	Intranasal	Cortisone acetate; cyclophosphamide; cyclophosphamide and cortisone acetate	Sarfati *et al.* 2002, Ibrahim-Granet *et al.* 2010, Wong *et al.* 2017, Morio *et al.* 2020
		Oropharyngeal	Triamcinolone acetate	Shepardson *et al.* 2014
		Intratracheal	Anti-Ly-6G / anti-Ly6C antibody	Shibata *et al.* 2014, Rolle *et al.* 2016
		Inhalation chamber	Cyclophosphamide and cortisone acetate	Sheppard *et al.* 2004, Steinbach *et al.* 2004
	Rabbit	Intratracheal	Hydrocortisone and cyclophosphamide; cyclosporin A and methylprednisolone	Chilvers *et al.* 1989, Berenguer *et al.* 1995
	Rat	Intratracheal	Cyclophosphamide and cortisone acetate	Schmitt *et al.* 1988, Chandenier *et al.* 2009
		Intrabronchial	Cyclophosphamide	Leenders *et al.* 1996
		Intrapulmonal	Cyclophosphamide	Habicht *et al.* 2002, Becker *et al.* 2006
Invasive aspergillosis, systemic	Guinea pig	Intravenous	Cyclophosphamide and triamcinolone acetate	Kirkpatrick *et al.* 2000
	Mouse	Intravenous	None; cyclophosphamide	Johnson *et al.* 2000, Sarfati *et al.* 2002
	Rabbit	Intravenous	Cyclophosphamide; cyclophosphamide and triamcinolone acetate	Patterson *et al.* 1988, 1991
Invasive aspergillosis, cerebral	Mouse	Intracranial, Intracerebral	Cyclophosphamide	Chiller *et al.* 2002, Clemons *et al.* 2012
	Rat	Intracisternal	None	Zimmerli *et al.* 2007
ABPA	Mouse	Intranasal, intratracheal or inhalation	None	Hogaboam *et al.* 2000, Kurup *et al.* 2001, Ramaprakash *et al.* 2009, Hoselton *et al.* 2010, Fei *et al.* 2011, Moretti *et al.* 2014
Aspergilloma	Rabbit	Intrapulmonal	None, but surgically induced artificial stenosis of the bronchus and the ligature of pulmonary artery	Sawasaki *et al.* 1967
Keratitis	Mouse	Intracorneal	None	Clark *et al.* 2016
	Rabbit	Intrastromal	Triamcinolone acetate (locally)	Komadina *et al.* 1985
Endocarditis	Guinea pig	Intravenous	None	Martin *et al.* 1997

[1] Selected references with detailed description of materials and methods.

developments: (i) equipment should evolve into smaller, more robust and easier to handle devices minimising expert dedication and maintenance cost; (ii) these should be coupled to a computer storage and artificial intelligence-driven computation system that will automatically process data into clinically meaningful results, this system could be on-site or securely accessed remotely; (iii) curated databases and pipelines should be developed that are directed to specific needs in the clinics; and (iv) expert personnel with bioinformatics and microbial genomics expertise should be incorporated into the clinical system. Some of these developments, particularly (i) and (ii), are progressing significantly thanks to the push of personalised medicine applications based on the human genome. However, (iii) and (iv), only partially overlap with other medical applications of genomics, and require a specific microbial genomics focus, and expertise. We envision that joint international efforts with the participation of regulatory authorities, researchers, and clinicians will help to make progress through pilot proof of concept studies and the standardisation of methodologies.

GENETIC TOOLBOX USED TO IDENTIFY ANTIFUNGAL RESISTANCE AND VIRULENCE DETERMINANTS

Inducing mating in *A. fumigatus* under laboratory conditions is a time-consuming process (O'Gorman *et al.* 2009), and studies of gene function have therefore heavily relied on site-directed mutagenesis (O'Gorman *et al.* 2009). However, the effect of the cell wall on the uptake of exogenous DNA and the low rate of homology driven repair (HDR) which ranges from 1–10 % in different strains makes gene manipulation of *A. fumigatus* an uphill road (Krappmann *et al.* 2006). Different techniques have been used in the attempt to find a balance between increasing transformation efficiency and limiting ectopic genome integration (Sánchez & Aguirre 1996, Sugui *et al.* 2005, Szewczyk *et al.* 2006); usually, polyethylene glycol-mediated transformation of protoplasts is preferred for site-directed mutagenesis (Brakhage & Langfelder 2002). Deleting essential players of the non-homology end-joining (NHEJ) pathway such as Ku70

Table 8. Consequences of antifungal resistance of *Aspergillus* species in murine models and *Galleria*.

Species/mutation	Antifungal	Main finding	References
Murine models			
A. fumigatus CYP51A (M220K, G54W)	Itraconazole, posaconazole	Systemic infection: resistance did not impact fitness and might increase virulence	Lackner *et al.* 2018
A. fumigatus CYP51A (M220K, G54W)	Itraconazole, posaconazole	IPA: resistance did not impact fitness	Valsecchi *et al.* 2015
A. fumigatus CYP51A (G448S)	Voriconazole	IPA: resistance was associated with reduced treatment efficacy	Krishnan-Natesan *et al.* 2012
A. fumigatus CYP51A (M220I, G54W, TR34/L98H)	Isavuconazole	Systemic infection: resistance was associated with reduced treatment efficacy in a dose-dependent manner	Seyedmousavi *et al.* 2015a
A. fumigatus CYP51A (M220K) and unknown	Caspofungin, posaconazole	Systemic infection: resistance was associated with reduced treatment efficacy in a dose-dependent manner	Arendrup *et al.* 2008
A. fumigatus	Posaconazole	IPA: resistance was associated with reduced prophylaxis efficacy	Seyedmousavi *et al.* 2015b
A. fumigatus	Itraconazole	Systemic infection: resistance was associated with reduced treatment efficacy	Denning *et al.* 1997a, 1997b, Dannaoui *et al.* 1999a, 2001
A. fumigatus CYP51A and Fks1	Posaconazole	IPA: resistance was associated with reduced treatment efficacy in a dose-dependent manner	Mavridou *et al.* 2010, Lepak *et al.* 2013
A. fumigatus	Anidulafungin, voriconazole	Systemic infection: resistance was associated with reduced treatment efficacy in a dose-dependent manner	Seyedmousavi *et al.* 2013a
A. fumigatus sequential isolates from CGD patient	anidulafungin, Posaconazole	Systemic infection: resistance was associated with reduced treatment efficacy	Arendrup *et al.* 2010
A. terreus	Voriconazole	Systemic infection: resistance was associated with reduced treatment efficacy	Salas *et al.* 2013
A. terreus	Amphotericin B, Itraconazole	Systemic infection: only itraconazole was effective	Dannaoui *et al.* 2000
A. flavus CYP51C	Voriconazole	Systemic infection: efficacy of voriconazole depended on drug exposure but correlated inversely with MIC	Rudramurthy *et al.* 2017
Insect models			
A. fumigatus CYP51A (M220K, G54E, G54W, TR/L98H)	Posaconazole, voriconazole	*In vitro* resistance associated with reduced treatment efficacy	Forastiero *et al.* 2015
A. fumigatus CYP51A (M172V, N248T, D255E, E427K, F46Y)	Voriconazole	Resistance did not impact virulence but was associated with reduced treatment efficacy	Garcia-Rubio *et al.* 2018a
A. fumigatus CYP51A (N248K/V436A, Y433N substitution)	Itraconazole, posaconazole, 8voriconazole	Resistance did not impact virulence but was associated with reduced treatment efficacy	Chen *et al.* 2019a
A. fumigatus CYP51A (G54, M220, TR/L98)	N/A	Resistance did not impact virulence	Gomez-Lopez *et al.* 2014
A. fumigatus sequential isolates from CGD patient	N/A	No association between virulence and resistance profile	Ballard *et al.* 2018
A. lentulus vs *A. fumigatus*	Voriconazole	*Aspergillus lentulus* could not be eradicated by voriconazole treatment in single and mixed infections	Alcazar-Fuoli *et al.* 2015
A. terreus; resistance mechanism not defined	Amphotericin B	*In vitro* susceptibility to L-AMB correlated with *in vivo* outcome	Maurer *et al.* 2015

(Krappmann *et al.* 2006) and Ku80 (da Silva Ferreira *et al.* 2006a) dramatically increases HDR frequency, so that 0.5–1 kb homology arms can efficiently mediate gene deletion (Krappmann *et al.* 2006). Although metabolic markers are available (Weidner *et al.* 1998, Xue *et al.* 2004) dominant selection markers are preferred, especially when using animal models, because position effects due to the marker integration site may ultimately affect fungal survival within the host (Liebmann *et al.* 2004, Greenstein *et al.* 2006). Commonly used markers (resistance towards hygromycin, phleomycin, or pyrithiamine) have been combined with site-specific recombinase systems to allow for marker recycling and targeting of multiple genes in the same background (Punt & van den Hondel 1992, Kubodera *et al.* 2002, Krappmann *et al.* 2005, Hartmann *et al.* 2010). Using a split-marker approach and a cloning-free fusion PCR strategy to assemble the transformation cassette further streamlined the process (Gravelat *et al.* 2012, Furukawa *et al.* 2020). Recently, the generation of a library of 484 transcription factor null mutants in a *Δku80 A. fumigatus* strain offered insights into the complex transcriptional regulation of azole response in this species (Szewczyk *et al.* 2006). Comprehensive libraries like this are a powerful resource to dissect pathways involved in

antifungal resistance, virulence, and nutritional versatility, which all contribute to the multifaceted pathogenicity of *A. fumigatus* (Ries *et al.* 2018, Pérez-Cantero *et al.* 2020). Nonetheless, using NHEJ-deficient strains in animal models calls for caution due to possible genome instability/sensitivity to abiotic stress, and reconstitution of a functional *ku80* gene is not feasible in large-scale efforts (Cairns *et al.* 2016). Moreover, dissecting the - still largely unclear - molecular basis of azole resistance in clinical isolates requires the ability to manipulate wild-type strains (van der Linden *et al.* 2015). Functional redundancy further muddies the water, and the sequential deletion of gene family members, albeit possible, is a painstaking process. These drawbacks can be overcome with the implementation of CRISPR-Cas9 editing technology (Fig. 4) (Morio *et al.* 2020). In 2016, microhomology-mediated end joining (MMEJ) was exploited to efficiently replace a Cas9-targeted gene with an *hph* cassette flanked by 28-bp homology arms in a NHEJ competent strain (Zhang *et al.* 2016). The same year, the expression of a ribozyme-flanked sgRNA from a RNA pol II promoter was combined with a split-marker approach to induce a single-nucleotide deletion in a *Δku80* strain containing an integrated tetracycline-inducible *CAS9* (Weber *et al.* 2017). Alternatively, Cas9-sgRNA ribonucleoproteins (RNP) can be used in conjunction with MMEJ to induce gene replacement with a *hph* marker in NHEJ competent strains (Al Abdallah *et al.* 2017). Multiplexing and protein-tagging have also been accomplished (Zhang *et al.* 2016). Recently, two gene-free intergenic safe haven regions were discovered in *A. fumigatus*, to which CAS9/sgRNA expressing constructs or selection markers may be directed, thus resolving potential position effects resulting from random ectopic integration (Pham *et al.* 2020). Notably, CRISPR-Cas9 finally allows high efficiency site-directed mutagenesis of wild-type strains. However, marker integration in the genome was originally required, either for replacing the target gene (Zhang *et al.* 2016, Al Abdallah *et al.* 2017) or for selecting the CRISPR elements (Weber *et al.* 2017). Last year, Ballard and colleagues (2019) tweaked the systems developed by Nødvig *et al.* (2015) and Weber *et al.* (2017) into a two-plasmid system for introducing SNPs into a clinical isolate without marker integration. Single-base CRISPR-Cas9 editing streamlined the association of amino acid substitutions with azole resistance in *A. fumigatus*, and opens up the way to the discovery of new clinically relevant and *CYP51A*-independent resistance mechanisms (Umeyama *et al.* 2018, Ballard *et al.* 2019).

Recently, the versatility of the CRISPR-Cas9 technology was exploited to dissect the molecular players of triazole resistance in a collection of *A. fumigatus* clinical isolates (Rybak *et al.* 2019). Cas9-sgRNA complexes were used to induce site directed mutagenesis and promoter replacement and to show that – surprisingly – mutations in *CYP51A* and overexpression of either *CYP51A*/*CYP51B* or the efflux pump *abcC* could not recapitulate the triazole MIC observed. Instead, the combination of CRISPR-Cas9 technology with a split hygromycin B marker approach confirmed that clinically occurring mutations in the sterol-sensing domain of the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) – observed in more than half of the clinical isolates examined – were indeed responsible for the triazole resistance observed (Rybak *et al.* 2019).

Non-editing applications developed in other fungi may also be adapted in the future to *A. fumigatus*, including the use of a catalytically dead Cas9 fused to transcriptional regulators to either activate (CRISPRa) or repress (CRISPRi) gene expression, which could address some of the shortcomings of RNA interference in this species (Cairns *et al.* 2016, Schultz *et al.* 2018). Disease outcome in patients at high risk of IA also depends on a timely diagnosis (Latgé & Chamilos 2019). The CRISPR technology may revolutionise the field of diagnostics with regards to nucleic acid detection and antimicrobial resistance profiling directly from clinical samples (Myhrvold *et al.* 2018, Quan *et al.* 2019). Overall, we may be at a turning point in the study of virulence and antifungal resistance, as the full potential of CRISPR-Cas9 removes long-standing roadblocks and re-shapes the *A. fumigatus* genetic toolbox.

APPLICATION OF MURINE AND INSECT MODELS TO IDENTIFY VIRULENCE DETERMINANTS AND ANTIFUNGAL RESISTANCE

In vivo infection models are essential for understanding pathogenesis, dissecting host-pathogen interactions, and identification of pathogen and host traits that contribute to susceptibility and disease development. For *Aspergillus* species, and *A. fumigatus* in particular, infection models for a variety of mammalian species, especially mice, rats, and rabbits have been used to model different types of disease caused by *Aspergillus* (Table 7). Technical aspects and the need for standardisation have been extensively reviewed recently by several authors (Paulussen *et al.* 2014, Desoubeaux & Cray 2017, 2018, Banfalvi 2018, Mirkov *et al.* 2019). Therefore, we will focus here on (i) differences between leukopenic and non-leukopenic mouse models of IA, as well as murine models of systemic aspergillosis, regarding the contribution of distinct fungal factors to virulence, (ii) the use of *in vivo* models, including insect models, to determine the impact of antifungal resistance, and (iii) the role of comorbidities.

Respiratory infection of leukopenic mice or mice receiving high-dose corticosteroids represent the most commonly used models to study IA (Desoubeaux & Cray 2017). These two models represent the two main groups of human patients at risk for IA, oncological patients undergoing stem cell transplantation or aggressive chemotherapy and patients after solid organ transplantation, respectively. Both models differ fundamentally in the role of the host response in pathogenesis: Leukopenia prevents substantial influx of immune cells into the lung and results in fungal growth unrestricted by the host responses, but driven by the fungal ability to germinate and acquire nutrients from the host tissue for growth (Stephens-Romero *et al.* 2005, Ibrahim-Granet *et al.* 2010, Kalleda *et al.* 2016). In contrast, large numbers of immune cells are recruited to the lungs of corticosteroid-treated mice, and while partially restricting fungal growth, immune cell recruitment contributes to pathogenesis in this model (Ibrahim-Granet *et al.* 2010, Kalleda *et al.* 2016). Thus, fungal factors required for growth in the lung generally impact virulence in both models, whereas determinants involved in the interaction with immune cells might only affect virulence in corticosteroid models. An example for this dichotomy is the secondary metabolite gliotoxin; while reduced gliotoxin production attenuates virulence in corticosteroid-treated mice, no significant effect was observed

in the leukopenic model (Kupfahl *et al.* 2006, Sugui *et al.* 2007, Spikes *et al.* 2008). Another example is the transcription factor DvrA that negatively regulates virulence in cell culture and the corticosteroid model but not in leukopenic mice (Ejzykowicz *et al.* 2010). IA furthermore differs from intravenous, systemic infection of mice that targets the liver, spleen and kidney (Jouvion *et al.* 2012, Paulussen *et al.* 2014). Organ-specific differences in nutrient supply are the likely reason why deletion of hcsA, involved in lysine biosynthesis, renders the mutant attenuated in IA but not systemic infection models (Schobel *et al.* 2010). These examples demonstrate the relevance of using different models for aspergillosis to fully dissect the contribution of fungal factors to host-pathogen-interaction.

While animal models have been and continue to be essential for studying pathogenesis of aspergillosis and identification of fungal virulence determinants, ethical concerns and practical considerations have led to the development and increasing use of invertebrate models, especially insects like adult *Drosophila melanogaster* and *Galleria mellonella* larvae (reviewed in Lionakis & Kontoyiannis (2012), Binder *et al.* (2016)). Important aspects of the innate immune response are conserved between insects and mammals (Medzhitov 2001, Müller *et al.* 2008, Sheehan *et al.* 2018), but insects lack lymphocytes and an adaptive immune system. While different levels of immunosuppression can be achieved by genetic manipulation in *Drosophila* (Neyen *et al.* 2014) or application of drugs (Chamilos *et al.* 2008), *Galleria* mutants are not yet available and non-treated insect hosts are commonly used. Furthermore, insect anatomy is dramatically different from mammals, and while both *Drosophila* and *Galleria* can be infected via different routes (Lionakis & Kontoyiannis 2012, Kavanagh & Sheehan 2018), it is not possible to mimic pulmonary infection. Thus, taking into account that the mode of immunosuppression and the route of infection determine the relevance of some fungal factors in mammalian aspergillosis models, results obtained in insect models cannot be directly translated to humans without confirmation in mammalian hosts. In fact, it yet remains to be determined which murine aspergillosis model the insect hosts resemble best, although infection of insects, which share several aspects with systemic infection of mice (Kavanagh & Sheehan 2018). Other limitations include the difficulties of infecting *Drosophila* with an accurate dose, and the necessity to maintain *Drosophila* at temperatures below 30 °C. Despite this, insect models have not only been used successfully to identify fungal virulence traits, but also in testing antifungal therapy.

Due to the lack of ethical restrictions, the short generation time, and limited space needed for insect models, they are ideally suited for comparison of a large number of fungal strains, semi-high throughput screening of novel antifungal compounds, and evaluation of combination therapies (reviewed in Kavanagh & Sheehan 2018 and Jemel *et al.* 2020). One limitation in many studies is however the lack of information on compound concentration at the site of infection. Although this has been addressed for *Galleria* in several studies (Forastiero *et al.* 2015, Maurer *et al.* 2015, 2019, Astvad *et al.* 2017, Kloezen *et al.* 2018), systematic analyses of pharmacokinetics in infected larvae (Jemel *et al.* 2020) and a comparison of larvae from different vendors are lacking. In addition, it remains unclear to which extent the metabolisation of antifungal compounds is comparable in insects and mammals. This is important for azoles, which target fungal cytochrome P450 oxidase and are also metabolised by the host (Sugar & Liu 2000, MacCallum & Odds 2002, Mavridou *et al.* 2010).

Animal models are essential for the development of therapies for aspergillosis, and especially murine models have been widely applied (reviewed in Paulussen *et al.* (2014), Lewis & Verweij (2017)). Both murine (reviewed in Lewis & Verweij (2017)) and insects (reviewed in Jemel *et al.* (2020)) models have more recently also been used to address consequences of antifungal resistance for treatment outcome (Table 8). Important developments are the use of coinfection with susceptible and resistant strains or species (Alcazar-Fuoli *et al.* 2015), the analysis of the consequences of resistance on fungal fitness *in vivo* (Lackner *et al.* 2018), and characterisation of strains evolved during chronic infection (Ballard *et al.* 2018).

One aspect that has not been extensively addressed in animal models for aspergillosis is the presence of comorbidities. Due to differences in anatomy and physiology, insect models are innately limited regarding options to model underlying diseases that affect humans at risk for aspergillosis. However, although murine models for various oncological and metabolic disorders, and infections that might occur in association with aspergillosis exist, they have not been applied to aspergillosis research. One example is aspergillosis in COVID-19 patients, discussed above. Murine models of influenza infection have been described (Thangavel & Bouvier 2014), as well as *Mycobacterium tuberculosis*, but a combination with aspergillosis has not yet been published. In fact, healthy young mice that are rendered immunocompromised shortly before infection might not adequately represent elderly patients with complex comorbidities receiving a variety of medications that might possibly interfere with antifungal therapy. They furthermore do not reflect the group of "non-immunocompromised" patients that develop IA (Stevens & Melikian 2011). The challenge in addressing comorbidities in animal models is that it makes the models significantly more complex and likely more difficult to standardise. Addressing this requires a combination of expertise for aspergillosis models and animal models for the respective comorbidity. This can likely be only realised by collaboration of groups across disciplines and might require a shift from the predominant use of murine models to other mammalian species like rabbits, which more closely reflect human immune response. Although laboratory mice are convenient to use and their genetic tractability allowed identification of distinct genetic polymorphisms associated with an increased risk for aspergillosis (Garlanda *et al.* 2002, Gresnigt *et al.* 2018), their small size limits repeated sampling and the course of the standard models for IA in mice is acute. For chronic types of aspergillosis, several models for ABPA have been developed (summarised in Takazono & Sheppard (2017)). In contrast, only a single model for chronic airway colonisation of immunocompetent mice has been described (Nawada *et al.* 1996, Urb *et al.* 2015, Wang *et al.* 2017) that has not yet been widely applied to study the chronic forms of aspergillosis. This model could be useful to address the possible role of *Aspergillus* in chronic pulmonary diseases such as cystic fibrosis (King *et al.* 2016a) and chronic pulmonary obstructive disease (COPD) (Gago *et al.* 2019). As mouse models for COPD and cystic fibrosis exist (Vlahos & Bozinovski 2014, Semaniakou *et al.* 2018), these could be combined with models of aspergillosis to gain more insight into pathogenesis.

In summary, a variety of *in vivo* models for aspergillosis using different host species have been described. Insect models are

valuable tools for questions that require a large number of strains or compounds to be screened and can be used to generate hypotheses later to be tested in mammalian models. Mice are the most widely used mammal to study aspergillosis, with practical advantages, but also some limitations. A variety of murine models have been established, that allow addressing different types of aspergillosis. For some aspects of aspergillosis, especially in the context of different comorbidities and also co-infections, it will however be necessary to refine existing models or to develop new high order models to adequately mimic human disease physiology and pathogenesis.

THERAPEUTIC DRUG MONITORING AND ITS ROLE IN ADJUSTING OPTIMAL AZOLE DRUG DOSAGE IN THE CLINICS

The pharmacokinetic/pharmacodynamic index that describes azoles activity against *Aspergillus* is the time/AUC (Lepak & Andes 2014) with an fAUC/MIC 0.33–25 corresponding to 50 % survival in mice or net stasis in fungal burden in lung (Lepak & Andes 2014). Preclinical and clinical studies have demonstrated exposure-effect and -toxicity relationships for voriconazole, posaconazole and itraconazole and targets for therapeutic drug monitoring (TDM) for treatment, prophylaxis and toxicity have been determined (Table 1), although most of them were based on low quality evidence (Ashbee *et al.* 2014). However, the benefit of TDM in treatment response as well as in adverse events of voriconazole have been demonstrated in a randomised controlled clinical trial (Park *et al.* 2012). Furthermore, TDM could prevent the development of resistance which usually occurs after 4 m (range 3 to 23 mo) of azole therapy with itraconazole, posaconazole (oral solution) and voriconazole (Arendrup *et al.* 2010, Camps *et al.* 2012c) as subtherapeutic levels may be associated with emergence of azole resistance (Howard *et al.* 2010, Moazam *et al.* 2020).

Given the large number of patients (40–60 %) having unpredictably subtherapeutic/undetectable levels of voriconazole, itraconazole and posaconazole (oral solution) (Park *et al.* 2012, Hoenigl *et al.* 2013a, 2014a, Prattes *et al.* 2016, Yi *et al.* 2017), TDM is recommended for most patients even for susceptible isolates (Arendrup *et al.* 2020). Patients for whom TDM is particularly recommended are those with erratic absorption (*e.g.,* due to non-compliance, mucositis, diarrhoea), distribution and elimination (*e.g.,* due to altered pathophysiology, genetic predisposition, insufficiencies, extracorporeal devices), potential drug interactions, poor response, difficult-to-treat infections (difficult sites of infection, resistant isolates) and those belonging to special patient population (*e.g.,* neonates, obese, elderly) (Hoenigl *et al.* 2014a, Lenczuk *et al.* 2018). Although the tablet/iv formulation of posaconazole provides sufficient exposure in most patients, the steady state with the current dosing regimens is reached after 7 d of therapy (Dekkers *et al.* 2016), although posaconazole levels (solution) obtained on day 3 to 5 showed high correlation with day 7 levels (Prattes *et al.* 2016). Considering the extra delay for TDM and dose adjustment, it may take > 2 wk until the new steady states are reached and verify that target levels are attained particularly for drugs with long half-lives like isavuconazole (Cornely *et al.* 2019b). Such a delay may be detrimental for invasive infections particularly in neutropenic patients since mortality increases from 41 to 90 % if effective antifungal therapy is delayed by 10 d (von Eiff *et al.* 1995). Population pharmacokinetic models (Hennig *et al.* 2006, van Iersel *et al.* 2018, Shi *et al.* 2019) and algorithms for early TDM (after 48 h) can be used to identify patients with subtherapeutic levels and speed up the dose adjustment process (Dekkers *et al.* 2016, Prattes *et al.* 2016). TDM can be coupled with monitoring fungal biomarkers like galactomannan (von Eiff *et al.* 1995) and PCR (Moazam *et al.* 2020) in order to optimise efficacy of azole therapy.

Azoles' efficacy is reduced by resistance which is often associated with high therapeutic failure rates (~90 %) (Howard *et al.* 2009, van der Linden *et al.* 2011). Most resistance strains have mutations in the *CYP51A* gene either tandem repeats in the promoter region (TR_{34}, TR_{46}, and TR_{53}) and/or single point mutations (mainly in codons 54, 98, 138, 220 and 448) whereas several other non-*CYP51A* gene mutations have been described (*CYP51B* overexpression, overexpression/modification of efflux pumps, mutations in other genes) (Dudakova *et al.* 2017). TDM could be used to optimise azole exposure against azole-resistant isolates. An exposure-MIC relationship has been demonstrated in experimental pharmacodynamic models (Siopi *et al.* 2014) and in a retrospective study (Troke *et al.* 2011) with a recommended TDM target for voriconazole of Cmin/MIC 2 for CLSI and 1 for EUCAST. The probability of a standard voriconazole dosing regimen to attain the pharmacokinetic/pharmacodynamic (PK/PD) target for an isolate with EUCAST MIC 2 mg/L is ~40 % requiring trough levels > 2 mg/L, which are feasible, whereas for isolates an MIC of 4 mg/L the probability drops to < 5 % requiring trough levels > 4 mg/L, which are usually associated with increased toxicity (Siopi *et al.* 2014). Similarly, the probability attaining the corresponding PK/PD targets of posaconazole and isavuconazole is very low for isolates with MICs > 0.5 mg/L (Seyedmousavi *et al.* 2014) > 2 mg/L (Espinel-Ingroff & Turnidge 2016), respectively. However, even if a clinically relevant PK/PD target has been determined, MIC-guided TDM approaches should consider the variation in MIC and in the PK/PD target. Isolates with voriconazole MIC 2 mg/L may harbour either the TR_{34}/L98H or the M220K/R/V CYP51A mutations with MICs 2–16 mg/L and 1–4 mg/L, respectively (Seyedmousavi *et al.* 2014, Arendrup *et al.* 2017c). Thus, an isolate with MIC of 2 mg/L may have a real MIC of 4 mg/L or higher depending on the underlying resistance mechanism and the corresponding MIC distribution of mutants leaving no space of optimisation via TDM. Given that resistant isolates have usually high modal MICs to itraconazole (> 16 mg/L), voriconazole (4 mg/L for TR_{34}/L98H and other mechanisms, > 16 mg/L for TR_{46}/Y121F/T289A) and isavuconazole (8 mg/L for TR_{34}/L98H, > 16 mg/L for TR_{46}/Y121F/T289A and other mechanisms though with wide variation) but lower to posaconazole (0.5 mg/L) (Meletiadis *et al.* 2012, Buil *et al.* 2018a, 2018b), posaconazole could be a good candidate for dose optimisation against azole-resistant aspergillosis. High dose posaconazole has been used to treat IA by azole-resistant isolates with posaconazole MIC 0.25–2 mg/L in patients (4/7 patients survived and 3/7 died from underlying diseases) (Mouton *et al.* 2018) and in dolphin after TDM (Bunskoek *et al.* 2017). A high dose regimen of isavuconazole (400 mg o.d.) has also been proposed for wildtype isolates with MIC 2 mg/L

(Espinel-Ingroff & Turnidge 2016) previously considered resistant but now belonging to the new category of area of technical uncertainty as isolates with posaconazole MICs 0.25 mg/L previously considered intermediate susceptible (Arendrup *et al.* 2020). An MIC-guided TDM has been proposed using the epidemiological cut-off value for the wild-type isolates and 4 × MIC for the non-wild type isolates (Mouton *et al.* 2018) limiting thus the role of TDM for azole dose optimisation against even the low-level azole-resistant isolates.

In addition to reduced efficacy, subtherapeutic levels may also be associated with emergence of azole resistance particularly in the setting of chronic aspergillosis or HSCT prophylaxis where long azole therapy is required (Steinmann *et al.* 2015, Moazam *et al.* 2020). Azole induced resistance has been described *in vitro* for isolates with non-*CYP51A* mutants and it has been associated with the overexpression of *CYP51B* gene and CDR1B efflux pump after exposure to itraconazole (Buied *et al.* 2013, Fraczek *et al.* 2013). Thus, it was hypothesised that azole-induced resistance which increases slightly the azole's MICs allows fungal survival until the development of stable resistance via mutation in target genes. Slight increases in MICs of posaconazole and itraconazole with ultimately development of resistance due to *CYP51A* overexpression has been reported in isogenic isolates recovered from patients after prolonged azole therapy (Arendrup *et al.* 2010). Those isogenic resistant isolates are unusually, harbour single point mutations (Camps *et al.* 2012c) or other non-*CYP51A* mutations (Howard *et al.* 2010). Subtherapeutic levels have been associated with the emergence of isogenic resistant isolates with non-*CYP51A* mutations and of non-isogenic resistant isolates harbouring the TR_{34}/L98H mutation (Howard *et al.* 2010). The PK/PD relationships and the clinical relevance of these phenomena should be further explored in order to estimate the potential role of TDM in preventing *in situ* development and *de novo* emergence of resistance.

Several methods have been used for TDM of azoles. Although chromatographic techniques (HPLC, LC-MS) are sensitive and specific, they are expensive, they have slow runtimes, they are not widely available and usually performed in central laboratories (Ashbee *et al.* 2014). Bioassays are cheap and simple to perform by each laboratory and can be adapted in different clinical settings including combination therapy (Siopi *et al.* 2016) but they lack specificity and measure total drug activity including any active metabolites. An immunoassay for quantitative measurement of voriconazole levels in serum samples has been developed and can be used in non-specialised centres (van der Elst *et al.* 2013). A dry spot blood technique has been developed for TDM of posaconazole, voriconazole and fluconazole (van der Elst *et al.* 2013). The DBS technique is simple, can be performed at home with finger prick blood, requires small sampling volumes, is less invasive and samples can be shipped at room temperature to the laboratory for analysis. A more innovative technique for TDM is the biosensor technology which provides real-time monitoring and dose adjustment of antimicrobials in a minimally invasive fashion (Rawson *et al.* 2018). An aptamer for sensing azole antifungal drugs has been developed moving forward this technology (Wiedman *et al.* 2017).

In conclusion, TDM of azoles is important in order to identify and optimise drug exposure in patients with subtherapeutic levels which are often observed in a large subset of patients treated particularly with voriconazole, itraconazole and the oral solution of posaconazole. Optimising azole exposure against low-level azole-resistant isolates in clinical settings is challenged by the precise and rapid determination of pathogen's MIC and the underlying resistance mechanism, the turnaround time of drug levels and the dose optimisation techniques for attaining steady target levels. Azole-induced resistance is an area that requires further research to understand the PK/PD relationships and define target levels. The development of bedside point-of-care tests for measuring drug levels is crucial for efficient TDM strategies. Because of azoles' complex pharmacology and non-linear pharmacokinetics, dose adjustment should be coupled with TDM early on therapy and frequent monitoring of drug levels depending on the disease and patient status.

TREATING AZOLE-RESISTANT ASPERGILLOSIS IN THE CLINIC

As discussed, the triazoles including voriconazole, posaconazole, and isavuconazole are recommended first-line antifungal therapy for IA, with itraconazole an option for the treatment of chronic pulmonary aspergillosis. Unfortunately, azole resistance has been an emerging problem over the last two decades, and treatment relies on either monotherapy with lipid formulations of amphotericin B or an echinocandin or combination therapy with an echinocandin or triazole plus lipid formulations of amphotericin B. Recent recommendations from the Netherlands (Schauwvlieghe *et al.* 2018a) outline the importance of primary treatment with lipid formulations of amphotericin B in settings where azole resistance is above 10 %, with a later switch to an azole if the isolate turns out to be susceptible to azoles. Several promising new antifungal agents are on the horizon that may serve an important role in the treatment of azole-resistant aspergillosis.

Amphotericin B appears inferior to the triazoles in the treatment of aspergillosis. In a large randomised trial comparing amphotericin B deoxycholate to voriconazole in patients with hematologic malignancy, the group receiving amphotericin B had lower rates of treatment response and survival at 12 wk compared to those who received voriconazole (Herbrecht *et al.* 2002). When treating azole-resistant aspergillosis with amphotericin B, lipid formulations are preferable to amphotericin B deoxycholate due to a decreased risk of toxicity and fewer infusion-related side effects (Hiemenz & Walsh 1996). In addition, a dose of 3 mg/kg of liposomal amphotericin B daily is preferable to 10 mg/kg daily given that no benefit has been seen and higher rates of nephrotoxicity associated with higher doses (Cornely *et al.* 2007). While the Infectious Diseases Society of America (IDSA) and European Conference on Infections in Leukemia (ECIL-6) do not specifically give recommendations on the treatment of azole-resistant aspergillosis, the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) gives liposomal amphotericin B a strong recommendation for the treatment of voriconazole-resistant IA (MIC = 2 mg/mL), a moderate recommendation for the treatment of chronic pulmonary aspergillosis and a strong recommendation for the treatment of aspergillosis with an MIC to voriconazole > 2 mg/mL (Hiemenz & Walsh 1996, Ullmann *et al.* 2018).

The echinocandins are not preferable for first-line treatment of IA, although they can be used in salvage therapy or combination therapy as discussed in more detail below. While caspofungin is

approved by the FDA for the treatment of IA, other echinocandins such as micafungin and anidulafungin are not FDA approved, although they have activity against *Aspergillus* species and appear equally efficacious to caspofungin. In one study of patients with IA who were intolerant to or refractory to other preferable agents, caspofungin showed utility as salvage therapy (Maertens *et al.* 2004). The ESCMID gives monotherapy with caspofungin or micafungin a marginal recommendation for the treatment of voriconazole-resistant aspergillosis (MIC > 2 mg/mL) (Ullmann *et al.* 2018) and a marginal recommendation (caspofungin) and a moderate recommendation (micafungin) for the treatment of progressive chronic pulmonary aspergillosis with triazole resistance (Hiemenz & Walsh 1996).

Combination therapy with liposomal amphotericin B and caspofungin has shown some benefit compared to monotherapy with liposomal amphotericin B for the treatment of IA. In a small prospective study of patients with hematologic malignancy, this antifungal combination was associated with favourable overall response and increased survival at 12 wk, although the survival difference was not statistically significant (Caillot *et al.* 2007). This combination has also shown promise in retrospective studies as salvage therapy (Aliff *et al.* 2003, Marr *et al.* 2004), although conclusions were limited due to the study design and small sample sizes.

Combination therapy with voriconazole plus an echinocandin has shown promise for the treatment of IA in some studies (Marr *et al.* 2004, 2015, Viscoli 2004, Singh *et al.* 2006), although again conclusions for this approach are limited based on study design and small sample sizes. In addition, none of these studies specifically looked at the treatment of azole-resistant aspergillosis. Still, the ESCMID gives combination therapy with voriconazole plus an echinocandin strong recommendation for voriconazole-resistant aspergillosis (MIC > 2 mg/mL) and voriconazole plus anidulafungin a moderate recommendation and posaconazole plus caspofungin a marginal recommendation for voriconazole-resistant aspergillosis with an MIC > 2 mg/mL (Ullmann *et al.* 2018).

NEW HORIZON

There are several new antifungal agents with promise for the treatment of azole-resistant aspergillosis. Fosmanogepix targets the highly conserved enzyme Gwt1 which catalyses an early step in glycosylphosphatidylinositol anchor biosynthesis, compromising cell wall integrity and fungal growth. This compound has been evaluated *in vivo* (Zhao *et al.* 2019) and in the murine model of IA (Gebremariam *et al.* 2019) and a phase II study is underway (ClinicalTrials.gov 2020b). Olorofim (previously F901318) is a novel member of the orotomide class that targets dihydroorotate dehydrogenase (DHODH), an important enzyme for pyrimidine biosynthesis. It has been evaluated *in vitro* (Buil *et al.* 2017a, du Pré *et al.* 2018), in the murine model of IA (Hope *et al.* 2017), and is currently being evaluated in a phase II clinical trial (ClinicalTrials.gov 2020a). Ibrexafungerp and rezafungin are other novel antifungals that show synergy when used in combination with azoles. Ibrexafungerp has shown to improve azole susceptibility for azole-resistant strains of *A. calidoustus* and *A. terreus* when used in combination. Rezafungin has shown some activity against azole resistant *Aspergillus* in a mouse model (Miesel *et al.* 2019). Further clinical data on these compounds is needed before determining their place in the treatment of azole-resistant aspergillosis.

FUTURE PERSPECTIVES

In the near future, there is a need for antifungal susceptibility testing of *Aspergillus* species to become the standard of care around the world. To accomplish this goal, more rapid and sensitive methods to detect ARAF need to be developed. Optimally, these methods would be applied as rapid tests and detect a broader spectrum of resistance markers directly in the clinical sample. Knowledge about the epidemiology of *Aspergillus* susceptibility patterns will represent a cornerstone for guiding the appropriate selection of antifungal prophylaxis and treatment. Given that more areas may be burdened with high rates of environmental triazole resistance, triazoles may not be universally recommended as primary antifungal treatment, but instead, treatment choice may depend on local epidemiology of ARAF. New antifungal agents, such as ibrexafungerp, that are currently in clinical stage evaluation with novel mechanisms of action may have central roles in treating these azole resistant infections, as well as species like *A. terreus* which are often less-susceptible to amphotericin B.

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