Mechanisms of Triazole Resistance in *Aspergillus fumigatus*

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Abstract

The ubiquitous fungal pathogen *Aspergillus fumigatus* is the primary cause of opportunistic mold infections in humans. *Aspergilli* disseminate via asexual conidia passively traveling through air currents to germinate within a broad range of environs, wherever suitable nutrients are found. Though the average human inhales hundreds of conidia daily, *A. fumigatus* invasive infections primarily affect the immunocompromised. At-risk individuals can develop often fatal invasive disease for which therapeutic options are limited. Regrettably, the global insurgence of isolates resistant to the triazoles, the frontline antifungal class used in medicine and agriculture to control *A. fumigatus*, is complicating the treatment of patients. Triazole antifungal resistance in *A. fumigatus* has become recognized as a global, yet poorly comprehended, problem. Due to a multitude of factors, the magnitude of resistant infections and their contribution to treatment outcomes are likely underestimated. Current studies suggest that human drug-resistant infections can be either environmentally acquired or *de novo* host selected during patient therapy. While much concerning development of resistance is yet unknown, recent investigations have revealed assorted underlying mechanisms enabling triazole resistance within individual clinical and environmental isolates. This review will provide an overview of triazole resistance as it is currently understood, as well as highlight some of the prominent biological mechanisms associated with clinical and environmental resistance to triazoles in *A. fumigatus*.

Keywords

Aspergillus; antifungal; triazole; resistance; ergosterol

Introduction

Among species of human filamentous fungal pathogens, one of the most pestilent is *Aspergillus fumigatus*, a saprophytic fungus extant in much of the world (Anastasi, Varese, and Marchisio 2005; Nierman et al. 2005; Denning 1998). This hyaline septate mold is ubiquitous, found in a plethora of natural climates and ecosystems as well as urban settings. While every human is estimated to inhale hundreds of conidia daily of this opportunistic

pathogen, invasive aspergilloses, (IA,) primarily affect humans with a compromised immune system. *A. fumigatus* represents a major cause of filamentous fungal infections among susceptible patient populations as well as affecting many domesticated animals including dogs, wild animals such as birds, and even insects (Seyedmousavi et al. 2015; Beernaert et al. 2009; Verweij, Chowdhary, et al. 2016; Bowyer, Bromley, and Denning 2020; Valdes et al. 2018). Infection culminates in a spectra of disease severity ranging from allergic conditions to invasive and disseminated aspergillosis (Groll and Walsh 2001; van der Linden et al. 2015). Overall mortality varies depending on the severity and type of underlying infection and on patient-specific factors such as severity of immunosuppression. Regardless of treatment, invasive disease poses a universally high mortality rate ranging from 30% to 90% for most patient types even with current antifungal therapies (Rybak, Fortwendel, and Rogers 2019; Denning et al. 2011; Verweij, Chowdhary, et al. 2016; Tekaia and Latge 2005; Falcone et al. 2011). Aspergillosis can be chronic, requiring continual and prolonged antifungal treatment lasting years in duration. In the recent past, invasive aspergilloses have seen a dramatic rise in incidence in multiple areas of the world. This epidemiological pattern of increasing incidence is largely due to prolonged survival of immunocompromised and immunosuppressed individuals, but is also linked to other shifting host-specific variables, changing environmental conditions, and to pathogenic adaptation to selective pressures (Brissaud et al. 2012; Groll et al. 1996; Groll and Walsh 2001; Lass-Florl and Cuenca-Estrella 2017; Verweij, Chowdhary, et al. 2016).

Aspergillus fumigatus is a saprophytic mold which primarily acts as a decomposer of organic matter (Latge 1999; Dagenais and Keller 2009). Aspergilli primarily reproduce asexually by producing many tiny structures known as conidia which are designed for dispersal through air (Levetin 2004). When some force such as weather, construction, landscaping, agricultural activity, animal activity or wind disturbs the mold's environment, conidia are dislodged. These structures can travel in contaminated material, air, and/or water and will germinate to grow wherever conditions and nutrients permit. Consequently, *A. fumigatus* is commonly found growing in both outdoor spaces and human structures. The fungus can be found subsisting on a wide range of ecological spaces such as within farmlands, greenhouses and in aquatic habitats including on surfaces including leaves, leaf litter, rotting wood, in soils and compost (Paulussen et al. 2017; Basenko et al. 2018; Ren et al. 2017; Zhang et al. 2017). Moreover, the species exists within indoor locales circulating in air as buoyant spores or in bioaerosols, on various surfaces, and within virtually any material or substance with adequate organic residues (Paulussen et al. 2017). *A. fumigatus* is even found growing within concrete and flooring material, especially following water damage (Andersen et al. 2011). For example, one source suggests that between 10% and 50% of buildings within North America and Europe exhibit levels of moisture suitable for fungal growth and between 15% and 40% of homes harbor mold within the structure (Andersen et al. 2011).

Conidia of *A. fumigatus* can be two to three micrometers in diameter, small enough to become lodged in lung alveoli. Both animals and humans commonly inhale conidia from *A. fumigatus*. The fungal spores are usually cleared by innate immune mechanisms. However, conidia that withstand host defenses can germinate, grow, and subsist by destroying surrounding tissue for nutrients (Dagenais and Keller 2009). The pathogen can spread and

disseminate to further sites via hyphal growth or angioinvasion and even conidiate inside a host within an aspergilloma (Eggimann et al. 2006; Dagenais and Keller 2009). While allergic bronchopulmonary aspergillosis (ABPA) and aspergillomas can occur, infections in healthy individuals are rare (Latge 1999).

One of the most common causes of invasive fungal infections in immunocompromised humans, *A. fumigatus* poses a threat to populations with predisposing conditions including solid-organ and stem cell transplant recipients, individuals undergoing certain chemotherapies, steroid use, those with acquired or inherited immunodeficiencies, severe liver disease, and with conditions affecting the lungs such as asthma, COPD, cystic fibrosis, bronchiectasis, sarcoidosis, respiratory infections such as influenza and very likely the COVID-19 coronavirus (Koehler et al. 2017; Lopez-Medrano et al. 2016; Steinbach et al. 2012; Wiederhold and Verweij 2020; Kosmidis and Denning 2015; Falcone et al. 2011; Resendiz Sharpe et al. 2018). In such individuals, infections typically begin in the lung following inhalation of fungal conidia but may then disseminate through tissue and bone to other locales. While the general inoculum required to cause disease and the incubation period for development of aspergillosis are not defined, infection likely depends on various aspects including the size of the fungal exposure, virulence of the strain, and host-specific factors (CDC 2019; Dagenais and Keller 2009; Paulussen et al. 2017). Excessive contact with conidia, for example, can lead to invasive disease for even immunocompetent humans (Kosmidis and Denning 2015). Moreover, while the link between fungal exposures and development of allergy such as ABPA, allergic *Aspergillus* sinusitis (AAS,) or *Aspergillus*-induced asthma (AIA) are clear, host specific factors such as genetics appear to determine susceptibility to allergic disease more than any external factor (Andersen et al. 2011; Shah and Panjabi 2016).

With developments and advances in modern medical strategies came the extended survival of immunocompromised populations and concurrent increases in certain diseases such as IA (Latge 1999). Multiple studies have attempted to approximate the global impact of aspergilloses and to discern any appreciable changes in incidence over time. Surveys have recently estimated the current burden of aspergilloses worldwide, approximating 4.8 million cases of ABPA, 3 million cases of chronic pulmonary aspergillosis (CPA,) and between 250,000 and 400,000 cases of IA annually (Brown et al. 2012; Bongomin et al. 2017; Bongomin et al. 2020; CDC 2019). IA occurs in up to 14% of heart transplant recipients, up to 7% of bone marrow transplant recipients, and up to 10% of hematological malignancy and lung transplant patients (Eggimann et al. 2006).

Unfortunately, within the United States, aspergillosis is not considered a reportable infection. Certain areas such as Seattle, Washington, where a children's hospital has had fourteen cases and lost seven patients since 2001 to nosocomial aspergillosis linked to contaminated air circulation systems, are moving to require reporting of aspergillosis to promote better responses to the disease (Staff 2019). However, the CDC does not require medical personnel to recount cases of confirmed or suspected disease in human patients, (though the CDC did prompt passive surveillance specifically for triazole resistant clinical isolates of *A. fumigatus* beginning in 2011) (Pham et al. 2014). Consequently, accurate numbers of active or past cases in the U.S. are difficult to procure and often depend on data

from retrospective studies. However, reasonable approximations for the weight of these infections on public health have been proposed based on available literature.

One of the first active evaluations in the U.S. to detect the burden of invasive fungal infections assessed data within the San Francisco Bay area from 1992 to 1993 and predicted the incidence of IA as 12.4 cases per million people annually. This study found aspergillosis to be the fourth most common invasive fungal infection (IFI) at that time. However, this study was limited to 45 hospital laboratories in a specific region of California (Rees et al. 1998). According to one study which evaluated information on a national hospital database, hospitalizations associated with IA rose by about 3% annually in the U.S. between the years 2000 and 2013, (approximately 169,110 IA-related hospitalizations total.) Using current census data, the same study estimated the rate of IA related hospitalizations to have increased in that time frame from about 32.8 cases per million people to 46 cases per million (Vallabhaneni et al. 2017). In a study utilizing available data from 2001 to 2006 to estimate the burden of IFI in the U.S., IA was the most common IFI detected (Kontoyiannis et al. 2010). Of the 16,200 hematopoietic stem cell transplant (HSCT) patients within the 23 participating centers of the study, 425 proven or probable cases of IA were found, with 187 confirmed as *A. fumigatus* specifically. The median time from transplant to onset of invasive aspergillosis infection was 99 days. For the 15,820 HSCT patients for which follow-up data was available, the total 12-month cumulative incidence (CI) of IA was 1.6%, though the incidence tended to show an increase over time. The 1-year mortality in this study among patients with IA was reported as 25.4% (Kontoyiannis et al. 2010). Unfortunately, details concerning patient treatment were unavailable for many of the cases and this study only evaluated HSCT patients. Another study of data from participating healthcare centers within Idaho and Utah from 2006 to 2016 found IA to be the third most common IFI, detecting 301 cases total, (40 confirmed specifically as *A. fumigatus*,) and estimating a mean annual incidence of 2.4 cases per 100,000 patients (Webb et al. 2018). This study reported a crude 1-year mortality for IA patients of 48.8% (Webb et al. 2018). The occurrence of IA among the healthcare centers remained stable throughout the period of study. However, this study only includes information within southern Idaho and Utah (Webb et al. 2018). The overall consensus in the literature is that incidence of IA both within the U.S. and across the globe has increased over the past thirty years, although approximations of the burden of aspergilloses on public health may vary depending on regional and local variables (Pfaller et al. 2018; CDC 2019; Hokken et al. 2019).

The list of approved therapies to combat aspergilloses include three specific classes of antifungals comprised of the polyenes, the echinocandins, and the triazoles. Primary therapy for IA begins with the triazoles voriconazole and isavuconazole, followed by the polyene amphotericin B (lipid formulations) and the echinocandin caspofungin. For patients who show no clinical response to primary treatment, salvage therapies also include other triazoles as well as amphotericin B, caspofungin, or combination therapy with multiple antifungals (Patterson et al. 2016). Antifungal prophylaxis, such as with the triazole posaconazole, is commonly used for at-risk individuals to preclude fungal infection. Moreover, surgery to remove infected tissue is sometimes required, such as for pulmonary aspergilloma (Walsh et al. 2008; Bellete et al. 2010; Patterson et al. 2016). Treatment approach depends largely on the type of infection and patient-specific variables. For example, patients with ABPA may

require treatment with anti-inflammatory corticosteroids to moderate lung function in addition to triazole antifungal doses sufficient to clear infection (Walsh et al. 2008; Denning, Pleuvry, and Cole 2013).

The triazole class of antifungals were introduced for medical use in the late 1990's and are currently utilized for therapy in human infections as well as certain animal species including birds (Beernaert et al. 2009; Hokken et al. 2019). This class of antimicrobial can be given orally with relatively low patient toxicity and effective activity against many fungal pathogens (Denning et al. 1989; Oakley, Moore, and Denning 1998; Heeres, Backx, and Van Cutsem 1984; George, Miniter, and Andriole 1996; Denning, Radford, et al. 1997; Verweij, Chowdhary, et al. 2016). Triazoles chiefly act to inhibit a key enzyme of ergosterol biosynthesis in fungi known as lanosterol 14α-demethylase, (Erg11 in many fungal species or the Cyp51 enzymes in *Aspergillus*) (Basenko et al. 2018; Arnaud et al. 2010). These compounds possess affinity for the substrate binding site of the fungal enzymes, thus blocking access for the natural substrate and inhibiting the synthesis of ergosterol, the key membrane sterol of fungi.

Unfortunately, the continued utility of the triazoles against *Aspergillus* spp. infections is now threatened by the global insurgence of triazole resistance. Studies indicate that triazole resistant invasive infection is positively associated with treatment failure and with patient mortality ranging from 30% to approaching 90% for some patient types (van der Linden et al. 2015; Lestrade et al. 2019; Falcone et al. 2011). Clinical cases of documented and confirmed *A. fumigatus* infections resistant to triazoles have originated from outdoor locales and within medical centers in various countries across the globe (Denning 1998; Groll and Walsh 2001; Chowdhary et al. 2015; Snelders et al. 2008; Snelders et al. 2009). While susceptibility of a strain to an azole antifungal is difficult to determine in-vivo, the common method by which antifungal activity is measured in-vitro involves the broth microdilution method, though methods such as disk-diffusion are also useful. Much of the literature employs minimum inhibitory concentration (MIC) values obtained through this method to approximate the susceptibility of an isolate to a given compound. Two standardized protocols exist whereby MIC values may be obtained; the European Committee on Antibiotic Susceptibility Testing (EUCAST) method, and a protocol provided by the Clinical and Laboratory Standards Institute (CLSI) (Alastruey-Izquierdo et al. 2015). While the CLSI has not yet set clinical breakpoints (CBP) of MIC values to mold-active medical triazoles for filamentous fungi, epidemiological cutoff values (ECV) based on the CLSI and EUCAST methods suggest that strains of *A. fumigatus* possessing an MIC for voriconazole or itraconazole above 1.0 µg/mL, isavuconazole above 2 µg/mL, or posaconazole above 0.25 µg/mL may be non-wild-type and should be considered for resistance (Alastruey-Izquierdo et al. 2015).

Investigations have attempted to uncover the prevalence of azole resistance among clinical and environmental isolates and estimate local or global incidence of resistant IA cases. A global surveillance study of 497 *A. fumigatus* isolates collected from 2008 to 2009 detected a total of 29 isolates with heightened triazole MIC values (Lockhart et al. 2011). A study of 1,972 isolates of *Aspergillus* from across the globe identified eight isolates of *A. fumigatus* with an MIC to itraconazole exceeding 8ug/mL (Pfaller et al. 2018). Another group

evaluated isolates submitted to the Mycology Reference Centre Manchester from 1992 to 2007 and then again between 2008 and 2009. Of the 519 isolates submitted to the center between 1992 and 2007, a total of 34 isolates were deemed resistant to at least one triazole, (possessing an MIC greater than 2μg/mL to itraconazole or voriconazole or >0.5μg/mL to posaconazole.) Interestingly, most of the patient isolates possessed cyp51A gene mutations, including TR34/L98H, G54(E,V, or R,), G138, M220(K or T,) and G448S, which are associated with triazole resistance in A. fumigatus and which will be discussed further in this review (Howard et al. 2009). At least 5 of the patient's deaths in this earlier study were blamed on progression of aspergillosis despite treatment with triazoles and, for some, efforts with alternative therapy. The frequency of resistance was only 1% from 1999 to 2004, but then rose to 8% from 2004–2007. The latter study found that resistance among isolates was 14% in 2008 and jumped to 20% in 2009 (Howard et al. 2009; Bueid et al. 2010). Another work published in 2008 reported similarly prominent cyp51A mutation occurrences among resistant isolates from the Netherlands, however in this investigation, the majority of isolates possessed only the TR34/L98H mutation rather than a variety of mechanisms (Snelders et al. 2008). Again in the Netherlands, a retrospective investigation into the prevalence of triazole resistance within patients in a university hospital ICU suffering from IA from 2011 to 2013 determined that of the 38 patients whose patient cultures grew A. fumigatus, 10 patients' isolates were deemed azole resistant. The 90-day mortality among the 28 patients with azole susceptible isolates was 82% (23 out of 28,) while mortality for the 10 patients with resistant disease was 100% (van Paassen et al. 2016). Isolates from the 10 patients with resistant IA harbored the cyp51A TR34/L98H and TR46/Y121F/T289A mutations. Microsatellite typing indicated the isolates were not likely related (van Paassen et al. 2016). In another survey for resistant A. fumigatus isolates within 16 participating hospitals and medical centers in the Netherlands published in 2016, the rate of resistance among tested isolates ranged from 5–10% overall, but was especially elevated within certain patient subsets where rates reached 30% (Lestrade et al. 2016). Resistant strains of A. fumigatus have now been identified within six of the seven continents (Dos Reis et al. 2019; Resendiz Sharpe et al. 2018).

Though the first *A. fumigatus* isolates found with resistance to triazoles originated in California in the late 1980's, some studies indicate that triazole resistance is less prevalent within the U.S. than in other regions of the world such as in Europe and the Netherlands (Walker et al. 2018). While the U.S. has fewer investigations than in European countries where active surveillance for triazole resistance has occurred regularly, some data is available for prevalence of resistant *A. fumigatus*. In a study of transplant recipients within the United States, occurrence of resistance within patient-isolated *Aspergillus* reached 7% (Baddley et al. 2009). Another study screened 1,026 clinical isolates of *Aspergillus* collected from 22 states in the U.S. from October of 2011 to October of 2013 for resistance to itraconazole (Pham et al. 2014). Of the 51 isolates which produced an MIC above 1ug/mL to the drug, one isolate with an MIC above 32ug/mL possessed an M220 mutation, (which, again, will be discussed later in this review.)

The true scope of triazole resistance in this pathogen and its effect on public health are likely underestimated due to the profound lack of regular screening for resistance by laboratories and centers both within the United States and around the world (Krishnan Natesan et al. 2012). Moreover, the frequency of negative patient cultures for this pathogen, despite

confident diagnosis of aspergillosis supported by serological diagnostics, renders detection of clinical resistance problematic (Denning et al. 2011). For example, systematic analysis of patient health records and matching autopsy records of adult patients admitted to ICU from 1966 to 2011 within the U.S. revealed that aspergillosis was one of the four most common potentially lethal patient misdiagnoses which likely resulted in patient death (Winters et al. 2012; Webb et al. 2018). To conclusively diagnose aspergillosis typically demands a positive culture from a normally sterile sample location as well as histological evidence for a patient *Aspergillus* infection, (though radiological scans, PCR-based assays, and blood galactomannan or beta-D-glucan assays can also aid diagnosis.) Consequently, infections with either resistant or wild-type *Aspergillus* strains are often misdiagnosed or otherwise undetected, making accurate assumptions of the true national and global impact of this pathogen difficult.

The origins, drivers, and effects of clinically and environmentally resistant isolates have been a subject of intense research since the first report of clinically resistant isolates correlated with patient treatment failure in 1997 (Denning, Venkateswarlu, et al. 1997). Unfortunately, due to the similarity in essential chemical structure and antifungal biomolecular activity, even agricultural fungicides can select for resistance to medical triazole drugs (Snelders et al. 2009; Schoustra et al. 2019; Faria-Ramos et al. 2014; Zhang et al. 2017). Indeed, this environmentally selected resistance is blamed for more clinically resistant patient infections than *de novo* resistance acquisition during extended medical triazole therapy (van der Linden et al. 2013). Regardless of the route of acquisition, mechanisms underpinning triazole resistance in *A. fumigatus* often come at no pathogenic fitness cost (Buil et al. 2019). Regrettably, much concerning the specific biomechanical causes, (whether genetically based or epigenetic,) clinical versus environmental derivation, and overall burden of triazole resistant *A. fumigatus* to public health within the United States and globally remains undetermined. This brief review aims to provide a succinct description of the selection of resistance in this species as well as to highlight some of the known environmental and clinical resistance mechanisms, especially those contributing to patient treatment failures and associated with increased mortality among infected immunocompromised patients.

Section One: The Rise of Resistance

The first instances of well-documented triazole-resistant *A. fumigatus* associated with treatment failure occurred in California during 1989–1990 and the results were published in 1997 (Denning, Venkateswarlu, et al. 1997). This study identified itraconazole resistant isolates from two separate patients with invasive aspergilloses undergoing triazole therapy. The isolates each exhibited an *in vitro* broth micro-dilution MIC value to itraconazole exceeding 16 μg/mL. *In vitro* resistance was confirmed to translate *in vivo* through experiments utilizing murine neutropenic models of infection (Denning, Venkateswarlu, et al. 1997). Differences in the phenotypes and sterol profiles of the resistant patient isolates pointed to the existence of at least two entirely separate stratagems of triazole drug resistance. One likely involved the increased expression, altered drug binding affinity, and / or enhanced activity of the fungal 14α- demethylase to counteract the drug's action whereas the other likely involved reduced intracellular accumulation of drug potentially as a result of

increased membrane transporter activity. Observations of this initial report have held true to the current view that multiple mechanistic strategies putatively underlie triazole resistance in *A. fumigatus* (Denning, Venkateswarlu, et al. 1997).

It is now understood that there are likely two general routes for selection of resistance in *A. fumigatus*: *de novo* selection within the host during therapy with triazoles or through selection in the environment (Verweij, Chowdhary, et al. 2016; Snelders et al. 2009; Buil et al. 2019; Verweij et al. 2013; Camps, van der Linden, et al. 2012; Hagiwara et al. 2014). Environmental saprobic fungi such as *A. fumigatus* are typically both phenotypically and genotypically plastic, a trait that allows for continual acclimation to ever-changing and generally adverse conditions (Groll and Walsh 2001). In turn, stressful stimuli within the environment often cause enduring adaptive responses and can select for beneficial and often heritable genetic or epigenetic changes within such organisms (Buil et al. 2019; Sniegowski and Gerrish 2010; Yona, Frumkin, and Pilpel 2015). Fungi can survive stress and outcompete other microbes within their environment through intracellular changes enabled by genetic alterations or via responses such as activation of efflux mechanisms or stress response pathways. Unfortunately, when demethylase-inhibiting fungicides are introduced into natural settings, as during agricultural work, fungi within these locales become exposed to the compounds and can readily adapt to the selective antifungal pressures (Groll and Walsh 2001; Chowdhary et al. 2015; Snelders et al. 2009). Though *A. fumigatus* is not a phytopathogen, the species acts as an important decomposer of organic matter, thriving wherever suitable nutrients and conditions are found including within outdoor environments and human-inhabited settings (Verweij, Chowdhary, et al. 2016). Multiple agriculturally employed azole fungicides are chemically similar and each possess the same primary mechanism of action against the fungal ergosterol biosynthetic pathway (Verweij, Snelders, et al. 2009; Stensvold, Jørgensen, and Arendrup 2012; Snelders et al. 2012; Zhang et al. 2017). Once introduced into water or soil, triazole fungicides can persist for at least several months (Ribas et al. 2016). Consequently, *A. fumigatus* present in such settings may become strained by these agricultural fungicides and counter with adaptive stress responses (Buil et al. 2019; Alvarez-Moreno et al. 2019). For example, three separate laboratory-based studies have reported the induction of target gene mutations similar to those found in clinical isolates via exposure to agricultural triazole compounds including tebuconazole, metconazole, bromuconazole, difenoconazole, propiconazole, hexaconazole and epoxiconazole. These compounds have been in use for agricultural applications and have been shown to select for strains possessing heightened MIC to the medical drugs voriconazole, itraconazole, posaconazole, or multi- or pan-azole resistance (Zhang et al. 2017; Snelders et al. 2012; Ren et al. 2017).

Exposures to resistant strains derived from the environment are blamed for a preponderance of the causal mechanisms identified within patient isolates (van der Linden et al. 2015; Chowdhary et al. 2015; Chowdhary et al. 2013; Verweij, Snelders, et al. 2009; van der Linden et al. 2009; Verweij, Chowdhary, et al. 2016; Verweij et al. 2013; van der Linden et al. 2013). However, the remainder of resistant clinical infections seem to result primarily due to prolonged or successive triazole treatments selecting for resistant fungi within the host (Howard et al. 2009; Chryssanthou 1997; Camps, Dutilh, et al. 2012; Verweij et al. 2002; Howard et al. 2013). Extended phases of antifungal treatments even spanning several months

can provide sufficient time for the development of resistant strains within the human host (Camps, Dutilh, et al. 2012; Denning et al. 2003). This is especially pertinent given that patients have been known to suffer from chronic aspergilloses requiring therapy exceeding 12 years in duration (Denning et al. 2003). A *de novo* resistant infection will likely no longer respond to treatment with the original triazole used and may additionally exhibit pan-azole resistance (van der Linden et al. 2015; Howard et al. 2009; Camps, Dutilh, et al. 2012; Verweij et al. 2002). Consequently, development of resistant infection during therapy can contribute to treatment failures and increased mortality (Dannaoui et al. 2001; Camps, Dutilh, et al. 2012).

It is, however, often difficult to prove the origins of a resistant strain and the occurrence of later infection with a resistant strain from the patient's environment cannot always be dismissed (Dannaoui et al. 1999). Analyses of the genomes of cultured strains are often utilized to propose the origins of a resistant infection (Dannaoui et al. 1999; Camps, Dutilh, et al. 2012). The presence of genetically diverse isolates possessing the same resistance mechanism in azole-naïve patients can imply isolates were selected for resistance within the environment (Wiederhold et al. 2016; Snelders et al. 2009; Walsh et al. 2008; Verweij et al. 2002). However, whole-genome sequencing experiments indicate *A. fumigatus* can undergo rather drastic genomic changes during invasive growth within the host niche, bringing such assumptions into question (Hagiwara et al. 2014). To provide clarity on isolate lineage, microsatellite typing has been employed to indicate whether serially collected isolates from a patient infection are "isogenic," having identical or very similar genotypes. As in the work by Hagiwara et. al., though related strains accumulated many separate mutations detectable through sequencing, they retained identical microsatellite results (Hagiwara et al. 2014). When genetic relationships are confirmed utilizing this method, a series of isogenic patient isolates which become resistant during extended patient treatment may suggest *de novo* resistance (Camps, Dutilh, et al. 2012). Regardless of the mode of resistance acquisition, infection with an azole-resistant strain of *A. fumigatus* is associated with an escalated cost of hospitalization and care, treatment failures, and increased mortality (Zilberberg et al. 2018; van der Linden et al. 2009; Lestrade et al. 2019; van der Linden et al. 2013). Resistant invasive *Aspergillus* infection elevates the observed mortality rate from about 50% for an azole-susceptible strain to approximately 90% for a resistant infection (van der Linden et al. 2011; Howard et al. 2009; Lestrade et al. 2019). Some of the most common resistance-associated mechanisms within both environmental and *de-novo* originated strains include triazole target or non-target alterations and are summarized in Table 1.

Section Two: Target gene-dependent resistance

Mutations affecting the target enzymes of triazoles, the fungal sterol 14α-demethylases, represent the most commonly identified mechanisms associated with triazole resistance in *A. fumigatus* (Snelders et al. 2010; Denning et al. 2011; van der Linden et al. 2013). *A. fumigatus* possesses two Cyp51 sterol 14α demethylase enzymes; Cyp51A and Cyp51B. Studies predict that Cyp51B has better binding affinity with triazole compounds. Aberrations have been identified within both genes encoding 14α demethylases of *A. fumigatus* isolates. However, only alterations affecting the *cyp51A* sterol-demethylase gene and its protein product have thus far been convincingly associated with clinically relevant

triazole resistance in this pathogen (Rybak, Fortwendel, and Rogers 2019; Ferreira et al. 2005). Target enzyme lesions have been found within both environmental and clinical isolates and have been recovered both from patients who have had extended contact with triazoles, as well as from infections of azole-naïve individuals (Wiederhold et al. 2016; Howard et al. 2006; Verweij, Chowdhary, et al. 2016). Sterol 14α-demethylase mutations have been identified in patient isolates and implicated in treatment failures among clinical patients in multiple countries (Wiederhold et al. 2016; Bueid et al. 2010; Howard et al. 2006; Chowdhary et al. 2015; van der Linden et al. 2013).

Depending on the type of mutation and its location in the genetic sequence, the resulting strain can exhibit a range of resistant phenotypes including a spectrum of triazole susceptibilities as well as single or pan-azole resistant qualities (Wiederhold et al. 2016; Howard et al. 2009; Chowdhary et al. 2015; van der Linden et al. 2013). Several principal stratagems exist whereby alterations in the coding sequence or upstream regulatory sequence of *cyp51A* enable resistance. The most commonly found Cyp51A mutations associated with triazole resistance occur at the gene sequence codons G54, L98, Y121 and T289, G138, and M220, with nonsynonymous mutations at position 98, 121 and 289 typically accompanied by a tandem repeat within the upstream genetic regulatory promoter region (Denning et al. 2011; Wiederhold et al. 2016). This category includes the mutation combinations TR34/L98H and TR46/Y121F, and TR46/T289A, which exist within patient derived and environmental isolates of *A. fumigatus* from multiple continents (Chowdhary et al. 2015; Verweij, Mellado, and Melchers 2007; Mellado et al. 2007; Snelders et al. 2008; Camps, Rijs, et al. 2012; Pham et al. 2014; Verweij, Howard, et al. 2009; Bader et al. 2013; Snelders et al. 2010; Wiederhold et al. 2016; Chowdhary et al. 2014; Snelders et al. 2009; Snelders et al. 2015; Stensvold, Jørgensen, and Arendrup 2012). Several of these will be discussed in more detail.

TR34/L98H is named such because of a tandem repeat of a length spanning 34 base pairs within the upstream promoter region of *cyp51A* and is also characterized by a substitution of a leucine at the 98th amino acid position to a histidine in the resultant protein (Gsaller et al. 2016b, 2016a; Bader et al. 2013; Snelders et al. 2010; Verweij, Mellado, and Melchers 2007; Abdolrasouli et al. 2015; Lockhart et al. 2011; Mellado et al. 2007; Rybak et al. 2019; Fuhren et al. 2015). This mechanism enhances transcription of the *cyp51A* gene, as well as altering the resultant protein enzyme's structure [Figure 1B] (Mellado et al. 2007). In *A. fumigatus*, the residue at position 98 lies near the first recognition site for the enzyme's substrate within the Cyp51A enzyme (Mellado et al. 2007; Snelders et al. 2011). Isolates possessing this mechanism tend to show reduced susceptibility to multiple medical triazoles including voriconazole, posaconazole, and itraconazole as well as the experimental azole compound ravuconazole and to multiple agricultural and industrial azole fungicides (Mellado et al. 2007; Mann et al. 2003; Snelders et al. 2008; Snelders et al. 2011). Cyp51A TR34/L98H has been well established as a functional cause of increased triazole resistance within human infections and is often associated with patient treatment failures and increased mortality (Pham et al. 2014). Strains with a TR34/L98H mechanism also retain virulence in a mouse model of invasive aspergillosis and possess no distinct defects in growth in comparison to wild-type isolates (Mavridou et al. 2013; Wang et al. 2018). The mechanism has been suggested to be the dominant causal contributor to azole resistance among both

environmental and clinical isolates of *A. fumigatus* and exists in samples from across the globe including the U.K., China, the Netherlands, Belgium, Austria, Norway, Denmark, Spain, France, Germany, Iran, India, and the U.S. (Gsaller et al. 2016b, 2016a; Bader et al. 2013; Snelders et al. 2010; Verweij, Mellado, and Melchers 2007; Abdolrasouli et al. 2015; Lockhart et al. 2011; Mellado et al. 2007; Chowdhary et al. 2012; Snelders et al. 2009; Chowdhary et al. 2015; Wiederhold et al. 2016; Fuhren et al. 2015; Pham et al. 2014). Among clinically resistant infections, TR34/L98H appears to be the result of both *de novo* resistance in azole-naïve patients and from environmental resistance involving strains selected in nature prior to human infection, the latter situation being more likely (Gsaller et al. 2016b, 2016a; Verweij, Mellado, and Melchers 2007; Verweij et al. 2002; Mellado et al. 2007; Snelders et al. 2008). Studies have illustrated the potential for this mechanism to arise in response to antifungal pressures. For example, duplication of the 34bp tandem repeat can be induced in an isolate possessing a TR34/L98H mutation following exposure to demethylase-inhibiting agricultural fungicides in a laboratory setting (Snelders et al. 2012).

While introduction of only the tandem repeat into a previously susceptible strain of *A. fumigatus* increases mRNA transcription in laboratory mutant strains, the introduction of both lesions simultaneously is required to recreate the phenotype of the clinically resistant strains (Mellado et al. 2007). Studies reveal that the 34bp repeat in the promoter region is bound by several important transcription factors which are known to affect the transcription of genes related to sterol biosynthesis as well as other pathways (Gsaller et al. 2016a, 2016b). One such transcriptional regulator, SrbA, binds to the repeated sequences in the *cyp51A* promoter in this mutant (Gsaller et al. 2016b). SrbA acts as a sterol regulatory element binding protein or "SREBP." Increased SrbA binding to the *cyp51A* promoter results in increased expression, thus likely exacerbating triazole resistance observed within these mutant strains (Gsaller et al. 2016b). Moreover, SrbA competes with the transcriptional repressor CCAAT-DNA binding complex (CBC) for DNA binding on the *cyp51A* promoter. Increased binding of SrbA blocks binding of the repressor resulting in de-repression of *cyp51A* transcription (Gsaller et al. 2016b).

The TR46/Y121F/T289A resistance mechanism also involves a tandem repeat of bases within the regulatory region as well as nonsynonymous mutations within the *cyp51A* gene (Wiederhold et al. 2016; Chowdhary et al. 2014; Snelders et al. 2015; van der Linden et al. 2013; Fuhren et al. 2015). The TR46/Y121F/T289A mechanism was initially found in the Netherlands within multiple voriconazole-resistant isolates of fifteen individual patients from six individual geographically distant hospitals, the earliest of which dated to December of 2009 (van der Linden et al. 2013). These patients suffered a high incidence of treatment failures and mortality through the study, especially among those given voriconazole. The same study sampled ten separate locations and recovered isolates possessing the same mechanism at six of the ten environmental locales (van der Linden et al. 2013). This resistance mechanism can spontaneously arise in response to antifungal pressure, as a study has reported generation of TR46/Y121F/T289A and G448S mutation combinations upon exposure of *A. fumigatus* strains to agricultural triazoles (Ren et al. 2017). The G448S mutation will be discussed in more detail later in this review. Another study found that introduction of the 46-basepair repeat and the Y121F mutation together without the T289A substitution into a previously susceptible strain appears sufficient to recapitulate the multi-

azole resistant nature of clinically obtained resistant isolates with the TR46/Y121F/T289A mutation pattern (Snelders et al. 2015). Isolates bearing this mutation typically have elevated MIC values for voriconazole and also attenuation of itraconazole and posaconazole susceptibility (van der Linden et al. 2013). The residue Y121 supposedly hydrogen bonds with the heme group within Cyp51A according to homology models, supporting the assumption of this residue as the more important site altering antifungal susceptibility when mutated [Figure 2A] (Snelders et al. 2015). In contrast, genetic introduction of either TR46 and the T289A mutation or the T289A mutation alone had little effect on susceptibility, though it may alter the enzyme's association with voriconazole (Snelders et al. 2015).

Mutations in the sequence for *A. fumigatus* Cyp51A at position G54 are also associated with cross-resistance to multiple triazole compounds including posaconazole and itraconazole (Chowdhary et al. 2015; Diaz-Guerra et al. 2003; Verweij, Howard, et al. 2009; Camps, van der Linden, et al. 2012; Mann et al. 2003; Nascimento et al. 2003; Bader et al. 2013; Mellado et al. 2007; Snelders et al. 2010; Pfaller et al. 2008; Rodriguez-Tudela et al. 2008; Krishnan Natesan et al. 2012; Denning et al. 2011). In the predicted tertiary structure of Cyp51A, the glycine residue at position 54 appears to interact directly with triazole drugs and is situated within the substrate access channel [Figure 2B] (Xiao et al. 2004; Snelders et al. 2010; Rybak, Fortwendel, and Rogers 2019; Diaz-Guerra et al. 2003). Multiple amino acid substitutions at glycine 54 are known including G54E, G54W and G54R (Mellado et al. 2007; Diaz-Guerra et al. 2003; Xiao et al. 2004; Tashiro and Izumikawa 2016). Replacement of the glycine residue at position 54 with an arginine (R) or a glutamate (E) in a previously azole-susceptible strain has been shown to increase MIC values to posaconazole by 30-fold while replacement with a tryptophan residue (W) produces a 250-fold MIC increase in laboratory mutants. These mutations also greatly increase MIC values to itraconazole but typically have no effect on *in vitro* voriconazole susceptibility (Xiao et al. 2004; Mann et al. 2003; Nascimento et al. 2003; Manavathu et al. 2000; Pfaller et al. 2008; Diaz-Guerra et al. 2003; Tashiro and Izumikawa 2016).

Another known mutation occurs at position G138 of the enzyme (Snelders et al. 2010; Manavathu et al. 2000; Xiao et al. 2004; Howard et al. 2006; Denning et al. 2011). The *cyp51A* G138C mutation was identified in a patient with chronic cavitary aspergillosis undergoing extended triazole therapy whose isolates simultaneously developed resistance to voriconazole and itraconazole (Howard et al. 2006). Isolates containing this amino acid substitution tend to be resistant to voriconazole but not to posaconazole according to both *in vivo* and *in vitro* measures of susceptibility or response to treatment (Xiao et al. 2004; Manavathu et al. 2000; Rybak, Fortwendel, and Rogers 2019). The residue at 138 is also situated within the ligand access channel near the heme cofactor of Cyp51A (Rybak, Fortwendel, and Rogers 2019). A G138R substitution likely disrupts the heme cofactor environment in the active site, disrupting the binding of voriconazole to the enzyme. The side-chain interactions of posaconazole may accommodate better association with Cyp51A and overcome the altered active site conformation (Xiao et al. 2004; Rybak, Fortwendel, and Rogers 2019). The G138 mutation has been spontaneously generated through exposure of *A. fumigatus* to triazoles in a laboratory setting, hinting at the potential for triazole antifungal stress to select for this mechanism. One such study induced the formation of a G138L mutation by exposing strains to itraconazole (Snelders et al. 2012).

Furthermore, one common lesion associated with azole resistance occurs at position M220 of the Cyp51A protein sequence (Mellado et al. 2004; Chen et al. 2005; Snelders et al. 2010; Mellado et al. 2007; Pfaller et al. 2008; Diaz-Guerra et al. 2003; Rodriguez-Tudela et al. 2008; Denning et al. 2011). Amino acid substitutions replacing the Methionine at residue 220 of the gene for Cyp51A have also been associated with an itraconazole resistant phenotype *in vivo* and elevated MIC values to itraconazole, voriconazole, and posaconazole *in vitro* (Mellado et al. 2004; Chen et al. 2005; Snelders et al. 2010; Mellado et al. 2007; Pfaller et al. 2008; Diaz-Guerra et al. 2003; Rodriguez-Tudela et al. 2008). This amino acid substitution has been detected within cultures recovered from diseased patients as well as through ultra-sensitive, real-time PCR assays for *A. fumigatus cyp51A* within patient samples which were not culturable (Denning et al. 2011; Howard et al. 2013; Howard et al. 2009). One such group of isolates were collected from a patient with chronic pulmonary aspergillosis (CPA) undergoing extended itraconazole treatment who developed a resistant infection. Upon the patient's death, researchers found that the individual's left lung had nearly entirely been destroyed; reduced to fibrotic tissue and interconnected aspergillomas (Howard et al. 2013). Individual strains cultured from separate aspergillomas displayed *cyp51A* mutations M220K or M220T and exhibited elevated MIC to itraconazole as well as to either voriconazole or posaconazole (Howard et al. 2013).

Finally, isolates possessing a G448S mutation have been associated with resistance to voriconazole and itraconazole (Krishnan Natesan et al. 2012; Bellete et al. 2010; Fuhren et al. 2015; Howard et al. 2009) and these mutants tend to remain susceptible to posaconazole (Xiao et al. 2004; Manavathu et al. 2000; Pfaller et al. 2008; Mellado et al. 2004; Howard et al. 2009; Krishnan Natesan et al. 2012). The residue at position G448 is also located near the heme cofactor in Cyp51A (Xiao et al. 2004). The mutation G448S has been established as a causal contributor to triazole resistance within the context of patient infections as well as an animal model of invasive pulmonary aspergillosis (Howard et al. 2009; Verweij, Howard, et al. 2009; Krishnan Natesan et al. 2012; Xiao et al. 2004; Manavathu et al. 2000). One individual developed a voriconazole resistant infection bearing the *cyp51A* G448S mutation after a year of treatment with voriconazole (Bellete et al. 2010). Though the patient was switched to lipid formulation amphotericin B and caspofungin therapy following the susceptibility testing results, in this case the patient ultimately died shortly after this modification of antifungal therapy. Sequencing of isolates recovered during the patient's extended care revealed that though the strains were identical according to microsatellite genotyping, the earlier collected strains which were recovered during treatment were susceptible to itraconazole and voriconazole while the later isolates possessed resistance to itraconazole, voriconazole, or both (Bellete et al. 2010). Among the four patient isolates and a reference strain, no other mutations existed in the *cyp51A* sequence in either the sense or antisense directions (Bellete et al. 2010). Laboratory mutant strains ectopically expressing *cyp51A* containing a G448S mutation introduced into a previously susceptible strain also recapitulate voriconazole resistance *in vitro* (Hagiwara et al. 2018).

Section Three: Target gene mutation-independent resistance

Often, sequence analysis of isolates with heightened MIC values unveils multiple strains which possess no Cyp51A-related lesions (Arendrup et al. 2010; Wiederhold et al. 2016;

Fraczek et al. 2013; Verweij, Chowdhary, et al. 2016; Buil et al. 2019). Indeed, a poorly understood shift from target enzyme-based mechanisms to alternative resistance mechanisms appears to be occurring in certain areas of the world (Fraczek et al. 2013). Common non-target gene mechanisms currently recognized include genetic and epigenetic occurrences such as alterations to transcription factor components, alteration of the sterol biosynthetic pathway, overexpression of specific drug efflux pumps or other means by which intracellular accumulation of triazole is reduced in the fungal cells, and enhanced adaptability due to biochemical or genomic plasticity (Gsaller et al. 2016a, 2016b; Camps, Dutilh, et al. 2012; Bellete et al. 2010). Selected genetic lesions which are not within the sequences or promoter regions for 14α demethylases have been well studied and convincingly associated with drug resistance and treatment failures in patients (Nascimento et al. 2003; Camps, Dutilh, et al. 2012; Fraczek et al. 2013; Ferreira et al. 2005). While a comprehensive list of all known or suggested azole resistance mechanisms is beyond the scope of this review, a selection of such methods will be discussed.

One such resistance mechanism involves a single point mutation in the protein coding sequence for a subunit of the fungal CCAAT-binding transcription factor complex, CBC, known as HapE in *A. fumigatus*. The CBC acts as a transcriptional repressor affecting the expression of genes such as *cyp51A* (Gsaller et al. 2016b; Camps, Dutilh, et al. 2012). The *hapE* P88L mutation was initially discovered within the sequences of multiple isolates obtained from a single patient with invasive pulmonary aspergillosis given extended triazole therapy (Camps, Dutilh, et al. 2012). In the case of this study, the patient ultimately failed therapy and succumbed to the invasive pulmonary infection. This outcome was very likely due to the resistant nature of the infection (Camps, Dutilh, et al. 2012). Microsatellite typing and sequencing analyses confirmed the isogenic nature of the early susceptible patient isolates of *A. fumigatus* and the later resistant samples. Analyses also revealed no changes within the gene sequences for Cyp51A and Cyp51B nor their promoter regions and only the *hapE* P88L mutation was able to recapitulate the invulnerable phenotype of the isolates. The P88L mechanism of resistance was reasonably verified as the causal mutation conferring a pan-azole resistant phenotype in the patient isolates through homologous gene replacement and sexual crossing studies with subsequent analyses of susceptibility and growth (Camps, Dutilh, et al. 2012). The repercussions of the P88L mutation involve loss of CBC activity in the mutant strain which results in de-repression thus allowing a higher level of *cyp51A* transcription within the pathogen, enabling resistance to triazole treatment [Figure 1C] (Gsaller et al. 2016b; Camps, Dutilh, et al. 2012).

A recent finding showing that altered sterol biosynthesis pathway activity underpins resistance in *A. fumigatus* clinical isolates came from work with the pathway rate-limiting HMG-CoA reductase, *hmg1* (Buil et al. 2019; Rybak et al. 2019; Hagiwara et al. 2018). One study which screened clinical isolates collected from multiple patients and from multiple medical centers in Japan for novel resistance mechanisms identified *hmg1* mutations among azole-resistant isolates (Hagiwara et al. 2018). The mutation S269F was present in each isolate which exhibited elevated triazole MIC collected from one patient with chronic pulmonary aspergillosis. The residue at S269 of *hmg1* resides within the beginning of the enzyme's predicted sterol-sensing domain (Hagiwara et al. 2018). However, several of the patient's isolates from this study also bore a G448S mutation in *cyp51A* and all resistant

isolates from the individual also possessed an A350T mutation in *erg6*, a major sterol methyltransferase (Hagiwara et al. 2018). This study further showed that ectopic expression of mutant *hmg1* and *erg6* alleles caused no shift in triazole MIC, suggesting that these mutations are not drivers of resistance (Hagiwara et al. 2018). However, another study positively linked mutations residing in the predicted sterol-sensing domain of *hmg1* within clinically resistant isolates to triazole resistance (Rybak et al. 2019). In this work, one of three mutations identified as unique to triazole resistant isolates or a wild-type *hmg1* allele were directly swapped with the native *hmg1* genomic locus of a previously susceptible laboratory strain (Rybak et al. 2019; Al Abdallah, Ge, and Fortwendel 2017). The resulting mutants with either an S305P or I412S mutation or an in-frame deletion of F262 showed increased MIC at least 4-fold to voriconazole, itraconazole, posaconazole, and isavuconazole, whereas the control wild-type allele matched the susceptibility of the parental strain. Furthermore, genetic knock-in of a wild-type *hmg1* sequence into the backgrounds of the hmg1-mutant resistant clinical isolates abrogated resistance (Rybak et al. 2019). These findings suggested that the sterol-sensing function of Hmg1 is an essential element for mediation of pathway activity and triazole susceptibilities in *A. fumigatus*. Further supporting this, sterol profiling confirmed an altered accumulation of precursors to ergosterol in strains carrying *hmg1* mutations, though the expression of *A. fumigatus* sterol demethylases was not abnormal (Rybak et al. 2019).

Apart from transcriptional or enzyme activity-based de-regulation of sterol biosynthesis, increased triazole export is another commonly identified mechanism of resistance. Multiple putative drug efflux transporters are transcriptionally upregulated in response to itraconazole treatment in *A. fumigatus*, implying a role in stress responses in wild-type strains (Fraczek et al. 2013). Drug efflux pump overexpression has been well-established in yeast species as a triazole resistance mechanism, but their contribution to resistance in molds has been somewhat less studied. However, multiple reports have suggested that constitutive overexpression of specific efflux pumps may be a mechanism of triazole resistance in clinical isolates of *Aspergillus* (Denning, Venkateswarlu, et al. 1997; Slaven et al. 2002; Nascimento et al. 2003; Rajendran et al. 2011; Fraczek et al. 2013; Nierman et al. 2005; Ferreira et al. 2005). The *A. fumigatus* genome contains genes encoding efflux transporters of the multidrug resistance (MDR) variety including major facilitator superfamily (MFS) and ATP-binding cassette (ABC) superfamily, few of which are currently associated with clinical or environmental resistance to azole compounds (Fraczek et al. 2013; Paul, Diekema, and Moye-Rowley 2013; Ferreira et al. 2005).

One study of resistant clinical isolates obtained both from patients who had prior triazole exposure and from azole-naïve individuals revealed clinically resistant strains overexpressing the gene *cdr1B* and which possessed increased MIC values to itraconazole, posaconazole, and/ or voriconazole (Fraczek et al. 2013). The *A. fumigatus* efflux pump-encoding gene *cdr1B,* also known as *abcB, abcC,* or *abcG1,* encodes an ATP-binding cassette (ABC) transporter (Fraczek et al. 2013; Rybak et al. 2019). Deletion of *cdr1B* in susceptible and itraconazole resistant isolates results in reduced itraconazole MIC values relative to controls, showing that loss of *cdr1B* increases susceptibility to the triazole (Fraczek et al. 2013; Hagiwara et al. 2016). Another study confirmed a genetic deletion of *cdr1B* within a triazole resistant background containing the TR34/L98H mutation resulted in

a significant decrease of resistance (Paul, Diekema, and Moye-Rowley 2013). Likewise, constitutive overexpression of the ABC transporter *atrF* also has been implicated in resistance (Slaven et al. 2002; Fraczek et al. 2013; Nascimento et al. 2003; Wang et al. 2018).

Several putative multidrug resistance (MDR) transporters in *A. fumigatus* have also been suggested to contribute to triazole resistance (Nascimento et al. 2003; Rajendran et al. 2011). The putative *A. fumigatus* efflux pumps Mdr1 and Mdr2, coded by the genes *mdr1* and *mdr2*, may underlie resistance (Tobin, Peery, and Skatrud 1997; Nascimento et al. 2003). In a study of 20 putative efflux transporters in *A. fumigatus*, levels of mRNA transcript for *mdr1* in the wild-type strain Af293 were found to be increased 14-fold over basal expression following four hours exposure to 1 mg/L itraconazole (Fraczek et al. 2013). Furthermore, gene deletion of *mdr1* in a wild-type strain of *A. fumigatus* results in increased susceptibility to itraconazole according to MIC results, indicating that this transporter is at least important in response to triazole-induced stress (Fraczek et al. 2013). Aberrations in the transcriptional levels of additional putative MDR efflux pumps in *A. fumigatus* have also been reported and have been suggested to play a role in resistance (Nascimento et al. 2003; Wang et al. 2018). A transcriptional analysis of a collection of isolates with high itraconazole MIC values identified strains possessing increased expression of a gene with homology to a major facilitator (MFS) transporter *mdr3* as well as to a gene with homology to an ABC transporter, *mdr4* (Nascimento et al. 2003). These strains displayed either constitutive expression of these transporters or heightened induction upon triazole exposure. However, genetic evidence confirming their roles in increased triazole MICs is currently lacking.

The same biochemical or genetic plasticity which enables fungi to adapt to stressors ranging from climate changes and nutrient availability to internalized toxins from competing microbiota can also allow for adaptation to antifungals (Verweij, Chowdhary, et al. 2016; Covo 2020; Hokken et al. 2019; Buil et al. 2019). Loss of genetic stability in fungal species such as *Candida albicans* and *A. fumigatus* has been associated with lessened susceptibility to triazole antifungals (Buscaino 2019; Selmecki et al. 2009). While frequent genomic changes can often result in inviable or weakened isolates under normal conditions, a moderate level of genetic instability can allow for selection of isolates inherently resistant to an adverse condition (Verweij, Zhang, et al. 2016; Dos Reis et al. 2018; Buscaino 2019). Genetic instability can result in ploidy changes, chromosomal rearrangement, and mutations in the genetic code. Mutations or disruption of genes encoding elements of DNA damage and cell cycle checkpoint pathways have been found to affect genomic stability and susceptibility to triazoles. For example, deletion of the genes encoding the AtmA and AtrA kinases in *Aspergillus fumigatus,* which are involved in the response to DNA damage and activation of DNA repair and cell cycle checkpoint pathways (Dos Reis et al. 2018). Moreover, loss of either gene atmA or atrA enhances adaptability to triazole stress to higher levels than wild-type, indicating that alterations in DNA damage or cell checkpoint players may affect evolvability and acquisition of resistant phenotypes (Dos Reis et al. 2018). The same group also reported that loss of the gene encoding DNA mismatch repair (MMR) protein MshA in *A. fumigatus* results in genomic instability that promoted enhanced evolvability upon exposure to posaconazole to resistant level MIC. Moreover, researchers detected the presence of mshA genetic variants among clinical and environmental isolates

(Dos Reis et al. 2019). This study mirrors studies of *C. albicans* strains lacking the related MMR protein MSH2 which similarly resulted in genomic instability and frequent occurrence of triazole resistant mutant strains, as well as a report that half of sequenced *C. glabrata* strains possess mutations in MSH2 which may contribute to resistance acquisition (Legrand et al. 2007; Healey et al. 2016). Moreover, study of *Cryptococcus neoformans* isolates which mutated at a high rate revealed strains bearing MSH2 mutations (Boyce et al. 2017). Although mutations that alter genetic stability have not been yet described as drivers of resistance in clinical isolates, these examples validate the possibility that genetic instability from aberrations in pathways such as DNA repair may enable acquisition of triazole resistance within *A. fumigatus*.

In addition to the genetic means outlined in this review, other mechanisms have been put forth as strategies by which *A. fumigatus* may undermine and survive the effects of triazole exposures. These include mitochondrial dysfunction, the formation of biofilms, epigenetic changes, RNA interference, alterations in intracellular networks, and impermeability to drug due to cell wall changes (Matthaiou et al. 2018; Bowyer, Bromley, and Denning 2020; Li et al. 2020; Hokken et al. 2019; Bromley et al. 2016; Valdes et al. 2018; Abdolrasouli et al. 2015). Many other stratagems likely exist which have yet to be detected or linked to resistance in environmental or clinical strains of *A. fumigatus* (Buil et al. 2019). While a discussion of every potential contributor to triazole antifungal resistance is beyond the scope of this review, such additional pathogen attributes may well impact the health of aspergillosis patients or alter the makeup of ecological niches and fragile microbiomes.

Conclusions

Fungal pathogens have become a considerable threat to human health as well as to animals and ecosystems globally. The rise of antifungal resistance only exacerbates this situation. The impact that *A. fumigatus* triazole resistance has on environmental microbiomes and patient treatment outcomes is only recently being appreciated. It is clear from the data collected thus far that drug resistance in *A. fumigatus* is associated with multiple distinct adaptive strategies. Often, the aberrant induction of gene transcription enables a strain to withstand drug activity by overproducing the drug's target enzyme or drug efflux mechanisms (Nascimento et al. 2003). Overexpression can also occur concomitantly with ORF mutations in the *A. fumigatus* gene encoding the triazole target, Cyp51A, to alter the binding affinity of triazoles to the enzyme. Such mutations can occur without detected changes in enzyme expression to confer triazole resistance. Moreover, resistance has also been associated with the overexpression of drug efflux transporters and various additional stratagems, including alteration of sterol biosynthesis in the mevalonate pathway, have been suggested to enable resistance to triazole compounds in this opportunistic pathogen. It is likely that we are only scratching the surface of the myriad mechanisms that filamentous fungi can utilize to subvert the activity of triazole compounds.

Although triazole resistance in *A. fumigatus* appears to be selected both during prolonged exposure to azoles within a host and in the environment by agricultural triazoles, the incidence of each route and the potential for common mechanisms acquired through each is still unclear. Increased surveillance for resistant *A. fumigatus* isolates in agricultural settings,

compost, indoor ventilation, and patient samples will be of high value in order to appreciate and respond to this threat to public health (Verweij, Mellado, and Melchers 2007; Zilberberg et al. 2018; Chowdhary et al. 2012). Adequate methods of surveillance and detection of pathogenic *Aspergillus* species, especially drug-resistant variants, are needed to predict the onset of allergic symptoms, nosocomial infections, and to guide treatment decisions in patient centers densely concentrated with at-risk individuals (Streifel et al. 1983; Rose 1972; Patterson et al. 2016). Unfortunately, studies on the fiscal and mortal burdens of *Aspergillus* diseases within the United States are few in number and often outdated (Zilberberg et al. 2018; Dasbach, Davies, and Teutsch 2000; Fraser et al. 1979; Steinbach et al. 2012). Improved surveillance to determine the prevalence of resistant strains, epidemiology, patient outcomes with resistant infections, contemporary effective treatment stratagems to combat invasive aspergillosis, and the occurrence of triazole resistance within both environmental samples and clinical patient isolates of *A. fumigatus* is essential (Steinbach et al. 2012; Neofytos et al. 2010; Patterson et al. 2016). This will enable effective study of the distribution and impact of resistant pathogens on ecosystems and human health (Fisher et al. 2012). Better understanding of the process of resistance development within *A. fumigatus* is also needed (Verweij, Chowdhary, et al. 2016). Probes into the process whereby strains become resistant may reveal stratagems to halt the rise of resistance in this opportunistic pathogen. Moreover, illuminating triazole-specific intracellular stress responses could identify novel targets and inform strategies to address resistant infections in novel stand alone or combination therapies (Snelders et al. 2008).

Acknowledgements

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Originality-Significance Statement:

This work addresses the understudied but significant issue of triazole antifungal resistance in the human and animal pathogen *Aspergillus fumigatus*. Though resistance within this species represents a threat to ecology and public health, much information concerning its origination and causes is limited, frustrating comprehension and progress in this area. This work concisely summarizes the process of triazole antifungal resistance progression within both patients and environmental settings as it is currently understood, as well as discussing multiple reported biological mechanisms associated with clinical and environmental triazole resistance in *Aspergillus fumigatus*.
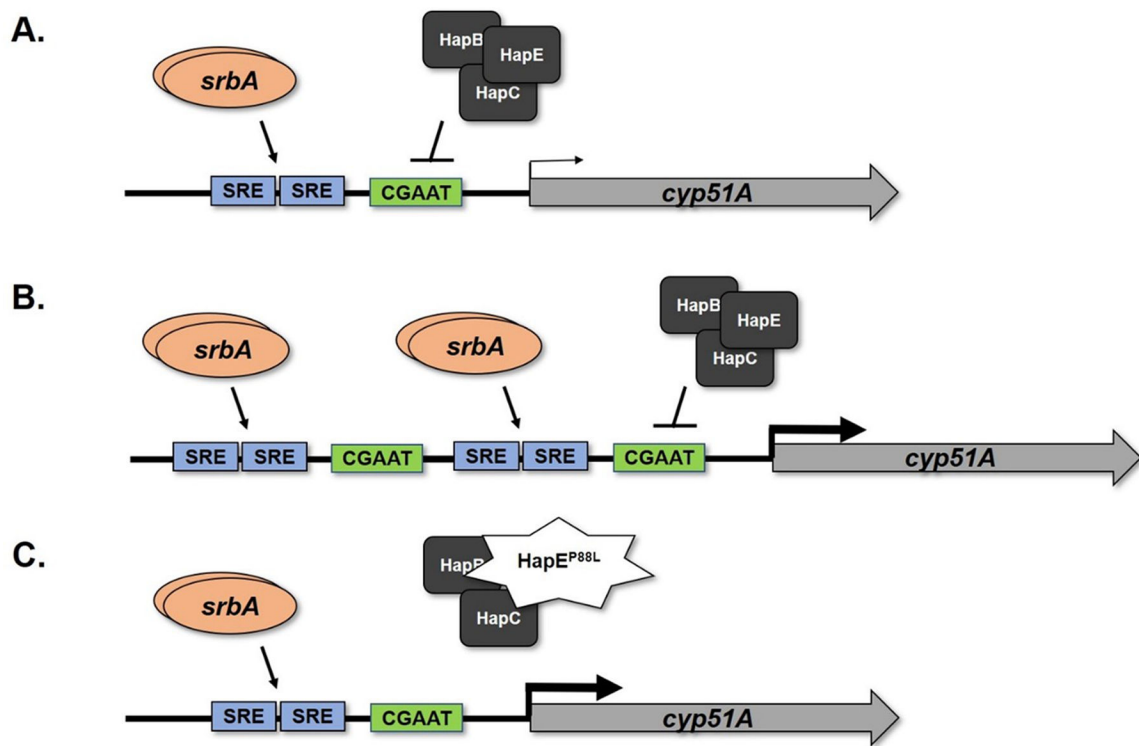
Figure 1. Illustration of genetic circuitry enabling enhanced *cyp51A* expression in isolates possessing one of two representative azole resistance mechanisms.
(A) The wild type configuration of the *cyp51A* promoter is shown. Under normal regulation, proper cyp51A expression levels are orchestrated by at least two transcriptional regulators. The activator, SrbA, binds to upstream sterol regulatory elements (SRE) to induce *cyp51A* expression, whereas the multi-subunit CCAAT-Binding Complex (CBC) binds to a conserved CGAAT motif downstream of the SREs to inhibit *cyp51A* expression. (B) Tandem repeat mutations of the *cyp51A* promoter wherein the SREs are duplicated, as occurs in isolates bearing the TR34 and TR46 mutations, lead to increased binding of SrbA and activation of gene expression. Although the CBC binding motif is also duplicated in these mutations, physical blockage of additional binding of the CBC complex occurs by the increased presence of the SrbA activator. (C) An illustration of a target gene-independent clinical triazole resistance mechanism involving a single mutation within the gene encoding a member of the heterotrimeric HAP complex, HapE. This mutation reduces binding of the entire CBC to its regulatory binding site in the *cyp51A* promoter. Note that, as in (B), reduced binding of the CBC complex results in loss of repression and consequently increased gene expression. Both representative mechanisms ultimately enable increased transcription of *cyp51A* mRNA, thus increasing the level of Cyp51A protein produced within fungi and enabling the biosynthesis of ergosterol.
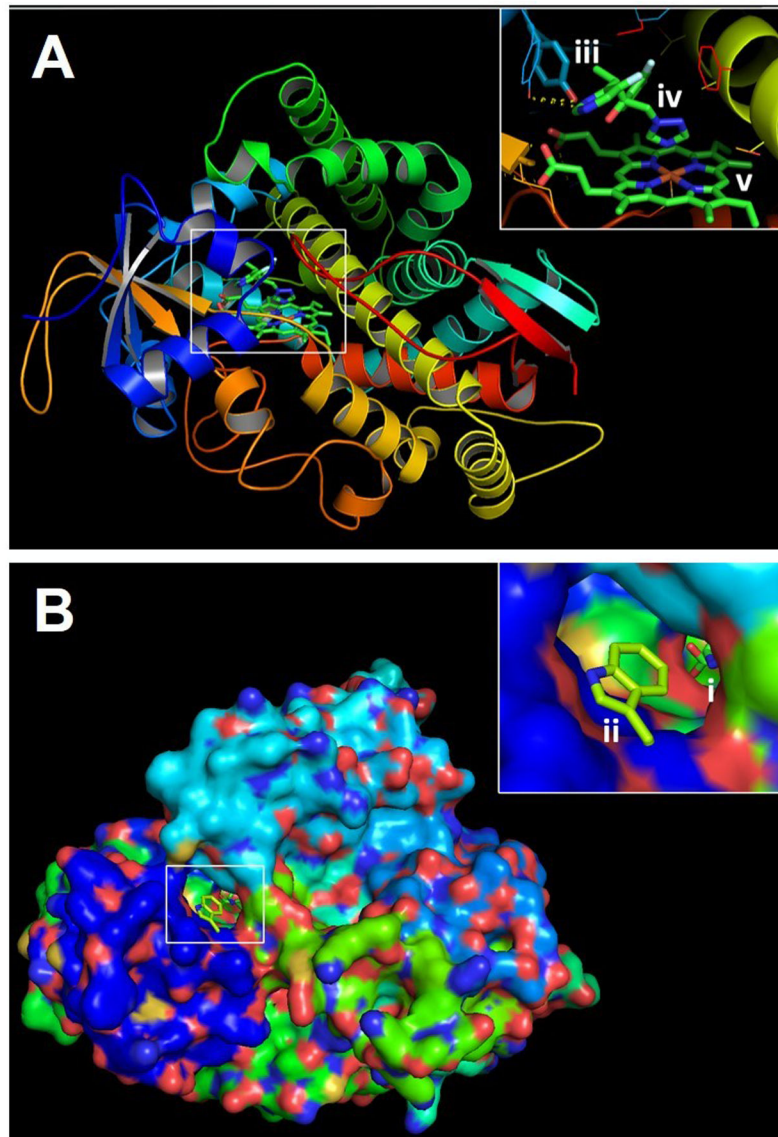
Figure 2. Protein model illustrating impact of *cyp51A* mutations on binding of the frontline anti-*Aspergillus* triazole, voriconazole.

PyMOL 2 was used to create three dimensional models of the *A. fumigatus* Cyp51A protein using the previously resolved crystal structure of the *A. fumigatus* Cyp51B as a scaffold. A) Three-dimensional ribbon model of Cyp51A. Inset depicts the Y121 residue (iii) which stabilizes the interaction between voriconazole (iv) and heme (v). B) Three-dimensional model of the surface of the Cyp51A protein. Inset depicts the ligand access channel and entrance to the Cyp51A catalytic site which is occupied by both heme and voriconazole (i). Also depicted is the G54W amino acid substitution (ii) predicted to interfere with the binding of the long lipophilic sidechains of itraconazole and posaconazole.

Table 1:

List of mechanisms associated with clinical triazole resistance in isolates of *A. fumigatus*.

Gene Name	Systematic Name Description	Alteration	Reference
cyp51A	Afu4g06890 14-alpha sterol demethylase	TR34/L98H	Mellado, E., et al., 2007 Snelders, E., et al., 2008 Gsaller, F., et al., 2016
cyp51A	Afu4g06890 14-alpha sterol demethylase	TR46/Y121F/T289A	Chowdhary, A., et al., 2014 Snelders, E., et al., 2015
cyp51A	Afu4g06890 14-alpha sterol demethylase	G54	Diaz-Guerra, T.M., et al., 2003
cyp51A	Afu4g06890 14-alpha sterol demethylase	G138	Manavathu, E.K., et al., 2000 Xiao, L., et al., 2004
cyp51A	Afu4g06890 14-alpha sterol demethylase	M220	Mellado, E., et al., 2004 Howard, S.J., et al., 2013
cyp51A	Afu4g06890 14-alpha sterol demethylase	G448S	Krishnan Natesan, S., et al., 2012 Bellete, B., et al., 2010
hapE	Afu2g14720 Sequence-specific CCAAT DNA binding transcription factor	P88L	Camps, S.M., et al., 2012
hmg1	Afu2g03700 Hydroxymethylglutaryl-CoA (HMG-CoA) reductase	S269F	Hagiwara, D., et al., 2018
hmg1	Afu2g03700 Hydroxymethylglutaryl-CoA (HMG-CoA) reductase	F262 deletion	Rybak, J.M., et al., 2019
hmg1	Afu2g03700 Hydroxymethylglutaryl-CoA (HMG-CoA) reductase	S305P	Rybak, J.M., et al., 2019
hmg1	Afu2g03700 Hydroxymethylglutaryl-CoA (HMG-CoA) reductase	I412S	Rybak, J.M., et al., 2019
cdr1B/ abcC	Afu1g14330 Putative ABC transporter	Overexpression	Fraczek, M.G., et al., 2013 Hagiwara, D., et al., 2016
atrF	Afu6g04360 Putative ABC transporter	Overexpression	Slaven, J.W., et al., 2002
mdr1	Afu5g06070 ABC multidrug transporter	Overexpression	Fraczek, M.G., et al., 2013 Nascimento, A.M., et al., 2003 Rajendran, R., et al., 2011
mdr2	Afu4g10000 ABC multidrug transporter	Overexpression	Fraczek, M.G., et al., 2013 Nascimento, A.M., et al., 2003 Rajendran, R., et al., 2011
Mdr3	Afu3g03500 Putative multidrug resistance protein	Overexpression	Nascimento, A.M., et al., 2003
Mdr4	Afu1g12690 ABC multidrug transporter	Overexpression	Nascimento, A.M., et al., 2003