ecdc
EUROPEAN CENTRE FOR DISEASE PREVENTION AND CONTROL

TECHNICAL REPORT

Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species

www.ecdc.europa.eu

ECDC TECHNICAL REPORT

Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species

This report of the European Centre for Disease Prevention and Control (ECDC) was coordinated by Niels Kleinkauf and Dominique Monnet.

Contributing authors
Niels Kleinkauf, Paul E. Verweij, Maiken C. Arendrup, Peter J. Donnelly, Manuel Cuenca-Estrella, Bart Fraaije, Willem J.G. Melchers, Niels Adriaenssens, Gert H.J. Kema, Andrew Ullmann, Paul Bowyer, David W. Denning

This report was sent for consultation to the European Food Safety Authority (EFSA).

Acknowledgements
ECDC would like to thank the members of the expert panel for the contributions and comments.

Suggested citation: European Centre for Disease Prevention and Control. Risk assessment on the impact of environmental usage of triazoles on the development and spread of resistance to medical triazoles in *Aspergillus* species. Stockholm: ECDC; 2013.

Stockholm, February 2013

ISBN 978-92-9193-444-7

doi 10.2900/76274

Catalogue number TQ-32-13-107-EN-C

Cover image courtesy of CDC, 2012.

© European Centre for Disease Prevention and Control, 2013

Reproduction is authorised, provided the source is acknowledged

Contents

Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
AML	Acute myeloid leukaemia
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary disease
CNS	Central nervous system
CPA	Chronic pulmonary aspergillosis
cyp51	Cytochrome P450 14-α sterol demethylase gene (gene coding for a demethylase enzyme in the ergosterol biosynthesis pathway)
DMI	Demethylation inhibitors
EBMT	European Group for Blood and Marrow Transplantation
EFSA	European Food Safety Authority (EFSA)
EORTC	European Organisation for Research and Treatment of Cancer
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FRAC	Fungicide Resistance Action Committee
GINA	Global Initiative for Asthma
HSCT	Haematopoietic stem cell transplant
IA	Invasive aspergillosis
ICU	Intensive care unit
MIC	Minimal inhibitory concentration
MSG	US Mycoses Study Group
PCR	Polymerase chain reaction
SAFS	Severe asthma with fungal sensitisation
TR_{34}/L98H	Resistance mechanism based on a 34-base pair tandem repeat in the gene promoter combined with a point mutation in the *cyp51A* gene at codon 98 leading to an amino acid change
TB	Tuberculosis

Executive summary

In recent years, triazole resistance in human *Aspergillus* diseases appears to have been increasing in several European countries. However, current data on the prevalence of resistance are based on a small number of studies which are only available from a few European countries. If present, triazole resistance can severely limit treatment options since alternatives, which are only available in intravenous form, have been shown to be associated with more side effects and poorer outcomes. Triazole resistance in *Aspergillus* spp. can evolve during therapy. Several point mutations, particularly in the *cyp51A* gene, have been associated with the development of resistance. Increasingly however, resistant isolates are also being detected in azole-naive patients. These isolates tend to have a particular genetic alteration consisting of a 34-base pair tandem repeat in the promoter coupled with a point mutation in the *cyp51A* target gene. This leads to an amino-acid substitution at codon 98 (TR_{34}/L98H) causing multi-azole resistance. In patients whose *Aspergillus* isolates have developed resistance during azole therapy wild-type isolates, closely related genetically to the resistant isolates, have regularly been recovered from samples taken before the start of therapy or during an earlier phase. To date however, no isogenic isolate with a wild-type phenotype has been recovered from patients infected with an *Aspergillus* strain carrying the TR_{34}/L98H genetic alteration. This suggests a possible environmental origin of the resistant fungus. This particular resistance mechanism has been observed most frequently in clinical isolates in the Netherlands where it has also been found in the environment. Moreover, the resistance mechanism has been demonstrated in clinical isolates in eight other European countries.

Azole fungicides are widely used for crop protection and material preservation in Europe. They protect crops from disease, ensure yields and prevent fungal contamination of produce. It has been proposed that triazole resistance has evolved in the environment and could be driven by the selective pressure of azole fungicides. Although evidence supporting this hypothesis is growing, the link between the environmental use of azole fungicides and the development of triazole resistance in *Aspergillus* spp. is not yet proven.

Triazole therapy has become the established treatment for invasive aspergillosis and is widely used in the treatment of allergic aspergillosis and chronic pulmonary aspergillosis. Antifungal therapy for invasive pulmonary aspergillosis is usually prescribed for a minimum of 6–12 weeks, but often may need to be continued for months depending on the period of immunosuppression. Treatment of allergic aspergillosis and chronic pulmonary aspergillosis may need to continue for years or even throughout a patient's lifetime. We estimated the burden of allergic, chronic and invasive aspergillosis using population statistics and published literature. Of the 733 million inhabitants in the European region[1] [1], at any one time 2 100 000 patients may be suffering from allergic aspergillosis and 240 000 from chronic aspergillosis, that would be an indication for antifungal therapy. For invasive aspergillosis, we have estimated an annual incidence of 63 250 cases, complicating multiple underlying conditions including leukaemia, transplantation, chronic obstructive pulmonary disease (COPD) and medical intensive care. The inability to treat these patients with triazoles due to multi-azole resistance would have significant impact on patient management and associated health costs.

Early and thorough investigation of this emerging public health problem is warranted in order to avoid the development and spread of resistance. This report examines current evidence for the environmental origin of resistance in *Aspergillus* spp. and makes recommendations for further steps to assess the risks and consequences of the environmental usage of azole derivatives. Improved surveillance of clinical isolates, including antifungal susceptibility testing, is the key to a better understanding of the magnitude of this emerging problem. Furthermore, the diagnosis of *Aspergillus* diseases needs to be improved and molecular methods allowing detection of resistance in culture-negative specimens must be further developed and implemented in laboratory practice. Finally, further environmental and laboratory studies are needed to confirm the environmental hypothesis.

Source and date of request

Request from the Netherlands, during the 24th Advisory Forum at ECDC, Stockholm, 8–9 December 2010, to organise expert meetings to support further investigation into the environmental origin of triazole resistance in *Aspergillus* diseases.

[1] For this report the European region was defined as the 27 EU Member States plus Albania, Andorra, Belarus, Bosnia and Herzegovina, Croatia, the Faeroe Islands, Iceland, Liechtenstein, the former Yugoslav Republic of Macedonia, the Republic of Moldova, Montenegro, Norway, the Russian Federation, Serbia, Switzerland and Ukraine.

Public health issue

Potential implications for human health through the environmental application of azole fungicides.

The objectives of this risk assessment were:

- To review current evidence on the environmental origin of triazole resistance in *Aspergillus* spp.;
- To assess the size and relevance of the problem for human health;
- To identify priorities for further investigation of this public health issue;
- To raise awareness and provide relevant authorities and decision-makers with options for action to control the emerging problem of azole-resistance in *Aspergillus* diseases.

Expert panel

Two meetings with experts from Europe and the USA were held: on 26 April 2011 in Amsterdam, the Netherlands and on 16 January 2012 at ECDC in Stockholm, Sweden. The participants are listed below.

Surname	First Name	Institution	Country
Adriaenssens	Niels	University of Antwerp	Belgium
Arendrup	Maiken C.	Statens Serum Institut	Denmark
Bowyer	Paul	University of Manchester	United Kingdom
Chiller	Tom	Centers for Disease Control and Prevention	USA
Cuenca-Estrella	Manuel	Spanish National Centre for Microbiology. Instituto de Salud Carlos III	Spain
Denning	David W.	University Hospital of South Manchester	United Kingdom
Donnelly	J. Peter	Radboud University Nijmegen Medical Centre	Netherlands
Fraaije	Bart	Rothamsted Research	United Kingdom
Kema	Gert H.J.	Wageningen University	Netherlands
Kortbeek	Laetitia M.	National Institute for Public Health and the Environment	Netherlands
Melchers	Willem J.G.	Radboud University Nijmegen Medical Centre	Netherlands
Ullmann	Andrew	University Hospital of Würzburg	Germany
van de Sande-Bruinsma	Nienke	National Institute for Public Health and the Environment	Netherlands
Verweij	Paul E.	Radboud University Nijmegen Medical Centre	Netherlands
Kleinkauf	Niels	European Centre for Disease Prevention and Control	EU
Monnet	Dominique	European Centre for Disease Prevention and Control	EU

External consultation

The final report was submitted to the European Food Safety Authority (EFSA) and its comments were taken into account.

Background

The term aspergillosis refers to a group of diseases which can result from *Aspergillus* infection, and host factors largely determine the type of clinical entity. It includes allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA), aspergilloma and the most severe form, invasive aspergillosis (IA). Some asthma patients with very severe asthma may also be sensitised to fungi-like *Aspergillus* (SAFS). *Aspergillus fumigatus* is by far the most common species in human *Aspergillus* infections, constituting more than 80–90 % of the isolates in most series [2-4]. In recent years, *Aspergillus* spp. and aspergillosis have been a major focus of clinical mycology because the number of patients with this disease has risen dramatically and the disease is difficult to diagnose and treat. The number of *Aspergillus* infections has increased because more patients are at risk of being exposed to this opportunistic pathogen and it is difficult to prevent the diseases it causes. Patients with established IA have poor outcomes. Successful therapy depends not only on an early diagnosis, which is difficult to establish, but also on the reversal of underlying host immune defects and the timely and effective use of antifungal agents. New diagnostic approaches have been introduced and antifungal agents have been developed for the treatment of aspergillosis. Particularly the newer azoles have become the mainstay of therapy and are the recommended first-line drugs in the treatment and prophylaxis of aspergillosis. Resistance in *Aspergillus* spp. has long been considered a rare event and has only been reported at low frequency, mainly in case reports. In recent years however, the situation has changed considerably. Several reports have been published and surveillance studies have been performed particularly in northern Europe, many of these indicating that there is an increase in the frequency of azole resistance in *A. fumigatus*. Studies from the United Kingdom and Spain have demonstrated an increase in the frequency of triazole resistance showing that the *cyp51* mutations found were due primarily to pressure under azole therapy [5-8]. At the same time there has been an increase in multi-triazole-resistant *A. fumigatus*, particularly in the Netherlands [9-11]. Here, the resistant strains were isolated from both azole-exposed and azole-naive patients, as well as from the environment. The majority of the resistant isolates carried a single resistance mechanism, based on a 34-base pair tandem repeat in the gene promoter combined with a point mutation in the *cyp51A* gene at codon 98 leading to an amino acid change (TR_{34}/L98H), and it was concluded that they were derived from the environment and may have evolved under the selective pressure of fungicides used in agriculture. The genetic alteration encoding this particular resistance mechanism appears to be spreading and has to date been detected in environmental samples in the Netherlands, Denmark, Norway, Italy and the UK (personal communication, D. Denning) and in patient isolates from nine European countries, India and China (Table 1) [5, 9, 12-19]. The emergence of multi-azole resistance in *A. fumigatus* may significantly impact on the therapeutic role of azoles in aspergillosis, as it would rule out the use of oral antifungals, leaving only the option of intravenous amphotericin B or echinocandins for life-threatening infections. The predictable consequence of resistance is increased morbidity, prolonged illness, a greater risk of complications, and higher mortality rates.

Table 1. Overview of countries that have reported TR34/L98H in *Aspergillus* spp. isolates from clinical or environmental samples

Country	Clinical samples		Environmental samples		Reference
	TR_{34}/L98H reported	Year[a]	TR_{34}/L98H reported	Year[a]	
Netherlands	+	1998	+ (cultivated soil, compost, seeds and leaves, air samples)	2002	[9, 77]
Norway	-	N/A[b]	+ (fjord water)	2001	[9]
Spain	+	2003	-[c]	N/A	[12, 78]
United Kingdom	+	2006	+ (rural area)	2011	[5], D. Denning, personal communication
Denmark	+	2007	+ (soil samples)	2009	[15, 78]
China	+	2008	-	N/A	[13]
India	+	2008	-	N/A	[16]
Austria	+	2009	-[c]	N/A	[71, 78]
Belgium	+	2009	-	N/A	[71]
France	+	2009	-	N/A	[71]
Italy	+	2009	+	2011	[18, 71]
Germany	+	2012	-	N/A	[19]

a Year of first recovery.
b N/A - not applicable.
c Soil samples were investigated, but TR34/L98H was not found.

In April 2011, the European Centre for Disease Prevention and Control (ECDC) arranged a first meeting to discuss the increasing problem of azole resistance in *A. fumigatus* with a particular focus on the possible environmental origin of resistance to medical triazoles. Public health experts, clinicians, agrochemical researchers and mycology reference laboratories were represented. A second meeting was held in January 2012. The current report represents a summary of the findings and proposals from the expert meetings.

Burden of aspergillosis in the European region

In 2010, the population of the European region (defined for this report as being the 27 EU Member States, plus Albania, Andorra, Belarus, Bosnia and Herzegovina, Croatia, the Faeroe Islands, Iceland, Liechtenstein, the former Yugoslav Republic of Macedonia, the Republic of Moldova, Montenegro, Norway, the Russian Federation, Serbia, Switzerland and Ukraine) was estimated by Eurostat to be 733 million inhabitants [1]. The numbers of patients in Europe suffering from aspergillosis is difficult to ascertain as figures have been obtained in a patchwork fashion, based on estimates from individual centres or countries. In the absence of proper registration, the best way to estimate the prevalence of aspergillosis is to rely on the validated population statistics for Europe (Eurostat), and apply the published frequencies of ABPA, SAFS, CPA and IA. Allergic fungal rhinosinusitis has not been considered in this estimation as it has not been demonstrated to respond to antifungal therapy. Such estimates are only an approximation, especially as the frequency of disease in each locality will vary, depending on aspects such as the occurrence of underlying diseases, the demographics of the population and other poorly recognised factors such as genetic risk. In addition, medical awareness is not uniform and diagnostic capabilities vary substantially. The use of antifungal prophylaxis and case ascertainment itself varies greatly according to the hospital and community medical setting.

Allergic aspergillosis

The principal allergic *Aspergillus* diseases are ABPA, SAFS (which is usually driven by *Aspergillus* spp., but other fungi may contribute) and allergic *Aspergillus* rhinosinusitis. Fungal rhinosinusitis due to *Aspergillus* was not considered in the following estimates as it has not been demonstrated to be responsive to azole therapy. Using the Global Initiative for Asthma (GINA) report and other data [20, 21] the number of adult asthmatics in Europe has been estimated at 35.5 million. The frequency of ABPA has only been estimated in referral populations and varies from 0.7 to 3.5% [22-26]. We have used the median of these figures: 2.5%. These data suggest an ABPA burden of just under 900 000 patients in Europe (Table 2).

Table 2. Estimated burden of aspergillosis, European region[a], 2010

Type of aspergillosis	Predominant diagnoses of population at risk	Population at risk (thousands)	Percent aspergillosis in population at risk	Annual burden of aspergillosis (thousands)	Reference
Allergic bronchopulmonary aspergillosis (ABPA)	Asthma	35 500	2.5% (0.7–3.5%)	887 (248–1242)	[20, 87]
	Cystic fibrosis	29	15%	4.3	[34, 35]
Severe asthma with fungal sensitisation (SAFS)	Severe asthma[b]	3 550	33% (25–50%)	1 172 (888–1 775)	[20, 87]
Chronic pulmonary aspergillosis (CPA)	Chronic obstructive pulmonary disease (COPD), tuberculosis, sarcoidosis, ABPA, pneumothorax	>13 600	1–10%	240	[37, 38, 87]
Invasive aspergillosis (IA)	Myeloid haematological malignancies[c]	44	7%	3.1	[39, 40]
	Lymphoid haematological malignancies[d]	N/A	N/A	3.1	[42, 57]
	Haematopoietic stem cell transplant (HSCT)	11.4	7%	0.8	[43, 57]
	COPD hospital admissions	2 830	1.2%	34.0	[45]
	Solid organ transplantation (SOT)	30	0.75%	0.23	[47, 49]
	Medical intensive care admissions	1 100[e]	2%	22	[52]

[a] In 2010, the European region (defined for this report as the 27 EU Member States, plus Albania, Andorra, Belarus, Bosnia and Herzegovina, Croatia, Faeroe Islands, Iceland, Liechtenstein, the former Yugoslav Republic of Macedonia, Republic of Moldova, Montenegro, Norway, Russian Federation, Serbia, Switzerland and Ukraine) had a population of 733 million inhabitants.

[b] Assumed to be 10% of asthmatic patients.

[c] Acute myeloid leukaemia, myelodysplastic syndrome and myeloproliferative disorders

[d] Acute lymphoid leukaemia, multiple myeloma, lymphoma and chronic lymphoid leukaemia.

[e] Assumed 30% of all ICU beds are for patients with medical diagnoses [34].

N/A - not available.

Asthma can be graded by severity, but definitions differ [27]. Using a 10% rate, there are an estimated 3.6 million adult Europeans with severe asthma, who account for ~70% of all asthma healthcare expenditure [28]. Alternative estimates based on total population indicate that 1–3% of the total population (adults and children) have asthma that is difficult to control [29]. Sensitivity to fungi in this population varies due to different testing approaches and variable performance of skin prick testing reagents and serum fungal-specific IgE testing. However, the most conservative estimate, using a limited panel of tests, put the percentage with fungal sensitisation at 33% [30], with some much higher figures also published [31-33]. This 33% rate of fungal sensitisation equates to a population of adult SAFS patients in Europe of 1 172 000.

Approximately 15% of patients with cystic fibrosis (CF) develop ABPA, and a larger number are colonised [34]. In the UK, CF occurs in 1 in 2 400 live births and a recent Europe-wide survey found 29 095 patients (2003–08) using multiple national registries [35]. This would imply ~4 300 patients with ABPA in the context of CF, although this probably overestimates the numbers as ABPA is less common in childhood.

Chronic pulmonary aspergillosis

Chronic pulmonary aspergillosis (CPA) is an uncommon complication of numerous pulmonary disorders including tuberculosis, sarcoidosis, ABPA, COPD, prior pneumothorax, cure of lung cancer, rheumatoid arthritis and occasionally occurring after IA [36]. Estimating the frequency involves ascertaining the frequency of the underlying diagnosis. We have attempted to do this for tuberculosis [37], ABPA [21] and sarcoidosis [38] which together account for about 50% of the total caseload seen in a national referral setting. The annual burden of tuberculosis-related CPA cases in Europe is estimated at 36 000, for ABPA 76 000 and for sarcoidosis 8 400; a subtotal of 120 400. Based on these assumptions, which accommodate a 15% annual mortality or surgical cure rate, approximately 240 000 adult Europeans have CPA at any one time (point prevalence).

Invasive aspergillosis

Invasive aspergillosis (IA) complicates haematological malignancy and its treatment relatively frequently. The commonest underlying disorder is acute myeloid leukaemia (AML), with an incidence of three per 100 000 population [39]. There will therefore be around 22 000 AML cases in the European region each year. Add to this an annual incidence of three per 100 000 for other myeloid malignancies, including myelodysplastic syndrome and myeloproliferative disorders, which are associated with a high risk of IA, and the number of high-risk haematological patients doubles to 44 000. Seven out of 10 cases are adults and the rate of occurrence of invasive mould diseases is reported to be around 8%, with IA accounting for nine out of ten cases – i.e. overall rate of occurrence 7% [40]. There are therefore likely to be 3 100 cases of IA among patients treated for myeloid malignancies in the European region each year. Approximately equal numbers of IA cases occur in patients with acute lymphoblastic leukaemia, multiple myeloma, lymphoma and chronic leukaemia [41, 42]. Hence, there are likely to be 6 200 cases of IA among patients treated for haematological malignancies and lymphoma in the European region each year.

In 2009, the European Group for Blood and Marrow Transplantation (EBMT) reported that there were 11 442 first-time recipients of an allogeneic haematopoietic stem cell transplant [43]. These patients are at similar risk of developing IA in the months immediately following transplantation [20]. Therefore assuming a 7% rate of occurrence [44] there would be 800 cases of IA per year, bringing the total number among those treated for haematological malignancies to at least 7 000 cases per year. At least three times this number are treated for IA each year, some on multiple occasions during different risk periods, and improved non-culture-based diagnostic testing indicates that the 7% rate of IA is a low estimate of the attack rate in this population.

IA as a complication of COPD is increasingly diagnosed , often post-mortem [45, 46]. Hospital admission rates for COPD vary in the UK from 124.7 to 646.5 per 100 000 population annually. If a mean of this (386) is used for the population in the European region as defined in this report, then approximately 2 830 000 COPD admissions would be expected in Europe annually. Of these, 1.2% develop IA (using longitudinal data for a large Madrid hospital [45], or approximately 33 950 patients. Most of these patients currently go undiagnosed and some are admitted to medical intensive care units (ICUs) (see below).

Solid organ transplantation and associated corticosteroid therapy are potent risk factors for the development of IA. Rates by transplant type vary and can be estimated individually or collectively. Accurate estimation of the risk in Europe is not possible with current data. Using UK data [47] and rebasing it for the main European countries indicates that about 30 000 solid organ transplants were done in 2009, but this figure is incomplete, and possibly an underestimate, especially with regard to renal transplants. Some countries, such as Belgium or Spain, have high organ donation rates, others much lower [48]. A prospective surveillance programme in the USA in 15 transplant centres, using the original EORTC/MSG criteria for diagnosis (which have some limitations for solid organ transplant recipients) established the overall rate of IA to be 0.75 % [49]. Using this figure, which almost certainly underestimates the rates, it is expected that there are ~225 IA patients in this population across Europe.

More patients with IA are being recognised in medical ICUs. There are estimated to be ~3.3 million admissions to ICU beds in Europe, with a seven-fold variation in bed numbers among countries (from 3.3 per 100 000 population

in the UK to 24.0 per 100 000 population in Germany) [50] and in number of patients per bed and per year (46–124 in Austria and Sweden, respectively) [51]. Based on US data it is assumed that around one third are medical patients [52]. The proportion of these patients who develop IA is 0.33–5.8 % [53]. For the purposes of estimation we have used a 2% rate, indicating that around 22 000 patients develop IA annually in European ICUs. There is also the possibility of double counting for some patients in the other risk groups above, which is difficult to fully adjust for, because of variable case-mix in different ICUs. On the other hand, some surgical patients also develop IA [54].

There are other patients at risk who develop IA, including those receiving corticosteroids and other immunosuppressive agents. It is not easy to estimate the numbers involved and these patients have therefore been omitted here.

Overall burden and implications of azole resistance

The overall mean burden estimate of all forms of aspergillosis in Europe is approximately 2 400 000 affected individuals annually (Table 2). These numbers do not include the estimated 1 870 000 individuals with allergic fungal rhinosinusitis which has yet to be demonstrated to respond to antifungal therapy. We estimate that 63 250 of these patients develop IA, a disease with a high mortality rate requiring urgent therapy. Slightly more than 2.3 million patients with allergic or chronic aspergillosis could potentially benefit from long-term oral azole therapy. Azole resistance is therefore potentially highly problematic for both groups of patients.

Clinical implications

Three classes of antifungal agents, the polyenes, the echinocandins, and the triazoles are available for the treatment of *Aspergillus* diseases. The choice of a certain compound is primarily based on the evidence obtained in clinical trials. A summary of the antifungal agents and their main use is shown in Table 3. Voriconazole is widely agreed to be the agent of first choice for IA. Its superiority as primary treatment for this condition over treatment with other classes of antifungals has been demonstrated in a number of clinical trials [55–57]. Only itraconazole has been studied for treatment of ABPA and SAFS.

Table 3. Classes of antifungals

Class	Drug	Route of administration	Indication	Comment
Triazole	Itraconazole	Intravenous/oral	Treatment of chronic *Aspergillus* diseases	
			Salvage therapy	
	Posaconazole	Oral	Prophylaxis of invasive fungal diseases	
			Salvage therapy	
	Voriconazole	Intravenous/oral	Primary therapy of invasive aspergillosis (IA)	
			Salvage therapy	
Polyene	Lipid formulations of amphotericin B	Intravenous	Primary therapy of invasive aspergillosis (IA) as an alternative choice for voriconazole	High costs
			Salvage therapy	
Echinocandin	Caspofungin	Intravenous	Salvage therapy	Exhibited low efficacy as primary therapy of IA in phase II trials

Failure of azole therapy

Case series consistently show high failure rates of patients with azole-resistant *Aspergillus* diseases to azole therapy. In patients with chronic *Aspergillus* diseases the failure rate was 89% [4], while in patients with azole-resistant IA 88% of patients had died within 12 weeks of obtaining a positive culture [10]. The clinical experience is supported by preclinical experimental data that show that the probability of azole treatment failure increases with increasing minimum inhibitory concentration (MIC) of the isolate [58-65].

The majority of azole-resistant *A. fumigatus* isolates are multi-azole-resistant. In a recent Dutch survey, 82 clinical azole-resistant isolates were all resistant to itraconazole (MIC > 2 mg/l), 79% were resistant to voriconazole (MIC > 2 mg/l) and 65% to posaconazole (MIC > 0.25 mg/l) (EUCAST breakpoints) [10, 66]. As a consequence, at best only a marginal role remains for azoles in the management of azole-resistant *Aspergillus* diseases.

Alternative treatment options

Clinical experience with alternative treatment regimens is limited. The *in vitro* activity of amphotericin B and caspofungin against azole-resistant isolates is similar to that against wild-type isolates, indicating that these agents may exhibit similar efficacy to wild-type isolates *in vivo* [62]. However, for both drug classes clinical evidence that these agents are efficacious in azole-resistant disease is lacking.

Experimental data are available for the combination voriconazole with anidulafungin in azole-resistant invasive aspergillosis [67]. These experiments indicate that the drug combination acts synergistically on mice infected with a voriconazole-susceptible isolate (MIC 0.25 mg/l). Although the combination showed better survival than voriconazole monotherapy in mice infected with a voriconazole-resistant isolate (MIC 4 mg/l), the drug interaction was indifferent. This raises concerns regarding the use of a voriconazole-echinocandin combination in isolates with high voriconazole MICs (i.e. 8 or 16 mg/l) as efficacy of the combination may then only be due to anidulafungin. The efficacy of anidulafungin monotherapy was only 45% in mice infected with the azole-resistant isolate as compared to that in mice infected with azole-susceptible isolates. Combinations of either caspofungin or micafungin with amphotericin B have been studied and shown not to be antagonistic, but are costly in clinical practice.

Preliminary results of liposomal amphotericin B efficacy in a non-neutropenic model of azole-resistant IA indicate that the efficacy of this polyene was not affected by the presence of the TR34/L98H or M220I resistance mechanism, compared to a wild-type control isolate (personal communication, P. Verweij). Clearly, more research is needed in order to guide decisions for treating patients.

Specific management problems

There are two patient groups for whom alternative therapy to azoles is very problematic. The first is treatment of azole-resistant central nervous system (CNS) aspergillosis, as voriconazole was found to have a better outcome than alternative agents. Case series show a 35% survival in CNS aspergillosis when treated with voriconazole, compared to approximately 10% in patients treated with a lipid formulation of amphotericin B, and 1% in patients treated with conventional amphotericin [68-70]. In patients with azole-resistant CNS aspergillosis an alternative drug with comparable efficacy to voriconazole is not available and successful management of patients is difficult. The second group is those with chronic *Aspergillus* diseases that require long-term antifungal therapy. As the azoles are the only class of drugs with activity against *Aspergillus spp*. that can be administered orally, ambulatory treatment would have to rely on intravenous therapy in cases of azole resistance. Increased costs, complications associated with intravenous access devices and the need for day admission in specialised healthcare institutions may create additional management problems.

Detection of resistance

The diagnosis of *Aspergillus* diseases can be difficult and cultures may remain negative. The detection of azole resistance further complicates the diagnosis and may result in delayed initiation of adequate antifungal therapy. In culture-positive patients *in vitro* susceptibility testing can be performed, which takes up to five days to complete. Rapid agar-based screening tools are being developed that may give an indication of resistance earlier [71]. In culture-negative patients, molecular tools can be used by detection of resistance mutations directly in clinical specimens. Although proof of principle has been reported, there are problems regarding the sensitivity of the assay, as the resistance hot spot gene is a single copy gene. Nevertheless, in one study resistance mutations were found in 55% of respiratory samples obtained from patients with chronic lung diseases [72]. This patient group is clearly different from those with IA, which is a rapidly lethal disease if treated inappropriately. As azole resistance may be present in azole-naive patients, the use of molecular tools to detect mutations directly on clinical specimens may enable rapid detection of resistance. However, there is an increasing list of mutations that are associated with azole resistance in *A. fumigatus* and in an increasing proportion of isolates the resistance mechanism is unknown. This limits the use of PCR-techniques and would require the development of new tools, such as sequence-based assays.

Routes of resistance development

Resistance has been shown to develop in patients with aspergilloma and cavitary lung disease who have been treated with azoles. Pathogenesis involves infection of the patient with an azole-susceptible *A. fumigatus* isolate that becomes resistant due to azole pressure. From patients receiving long-term azole treatment, azole-resistant *A. fumigatus* isolates have indeed been described to contain numerous point mutations including those in codons 54 and 220 of *cyp51A* [5-8, 73]. This confirms the general thought that these resistance mechanisms are gained under high azole pressure within the patient. Substantial diversity of *cyp51A*-related resistance mechanisms has therefore been found which was shown to be associated specifically with azole therapy [5]. Importantly, spread of resistance is highly unlikely in IA, because person-to-person transmission is very uncommon and a patient will either respond to treatment (precluding spread of the isolate) or the treatment will fail. In the latter case, the (resistant) fungus might survive, but its spread directly to another patient or through the environment seems highly unlikely. Patient-to-patient transmission of IA is extremely uncommon, and reports have only been published of two clusters: transplantation of a contaminated allograft and a surgical wound infection [74, 75].

Another route of resistance development might be through exposure of *A. fumigatus* to azole compounds in our environment. Exposure of saprophytic fungi to azole compounds could take place in agriculture, where such compounds are commonly used for plant protection. The fungicides are applied repeatedly over a long period of time and could thereby create a persistent pressure of azole compounds on saprophytic fungi. During the saprophytic growth of *Aspergillus* spp. in the environment, the asexual and sexual reproductive modes not observed in patients with acute IA may promote the efficient spread of particular mutations well adapted to the selective pressures exerted by azole compounds. Furthermore, an environmental route of resistance development would be in line with the finding of primary IA due to azole-resistant *A. fumigatus* in azole-naive patients. Breakthrough aspergillosis in patients on azole prophylaxis would also be consistent with an environmental route: patients would inhale both azole-susceptible and azole-resistant conidia, but the resistant conidia would have a selective advantage, thus allowing their germination in the lungs and subsequent invasive disease. This route of resistance will be further elaborated in the next paragraph.

Evidence for the environmental route

The following observations support an environmental route of azole resistance development:

- **Azole resistance observed in azole naïve patients.** A recent Dutch culture-based survey in seven university medical centres showed that 64% (14/22) of patients with azole-resistant *Aspergillus* spp. disease known to have been exposed to azole were azole naïve at the time the resistant isolate was cultured [10]. As patients are exposed to *Aspergillus* spp. primarily through inhalation of airborne conidia, this observation suggests that azole-resistant spores are present in the air. Patients are thus exposed to azole-susceptible and azole-resistant spores and either could go on to cause *Aspergillus* disease in a sensitive host. Treatment with azoles could favour the germination of those conidia that harbour a resistance mechanism.
- **A dominant resistance mechanism is found in the Netherlands.** In the Netherlands the dominant resistance mechanism consists of a substitution at codon 98 (L98H) and a 34-base pair tandem repeat in the gene promoter (TR_{34}/L98H). Two surveillance studies have reported this specific resistance mechanism in >90% of clinical isolates found to be itraconazole resistant [9, 10]. This contrasts with observations in patients with a documented patient route of resistance development, characterised by a wide diversity of mutations in consecutive cultures from different colonies recovered from a single specimen. As *Aspergillus* diseases are not contagious, patient-to-patient spread of isolates (and resistance mechanisms) is highly unlikely, the finding of a dominant resistance mechanism is not in keeping with the patient route.
- **The presence of two genomic changes (including a tandem repeat).**The dominant resistance mechanism found in the Netherlands consists of two genetic changes, while isolates that have become resistant through patient therapy tend to have a single mutation as underlying resistance mechanism [76]. The development of a complex resistance mechanism during azole therapy may be less likely. Furthermore, a tandem repeat has been found as resistance mechanism in phytopathogenic fungi that have become resistant to azole fungicides.
- **Isolates harbouring the TR_{34}/L98H resistance mechanism are found in the environment.** Several studies have shown that *A. fumigatus* isolates from environmental samples may be resistant to medical triazoles. This has been found in soil samples obtained from flower beds, compost, leaves and seeds purchased from a commercial garden centre [77]. The resistance mechanism found in the majority of isolates cultured from the environment is TR_{34}/L98H. Moreover, TR_{34}/L98H isolates of environmental origin have been reported in the Netherlands, Denmark, Norway, the UK and Italy [77, 78]. Genetic typing of TR_{34}/L98H isolates of clinical and environmental origin using microsatellites showed that TR_{34}/L98H isolates clustered and were less diverse than wild-type controls and azole-resistant isolates with point mutations as the underlying resistance mechanism [79].
- **Triazole fungicides used in agriculture have a similar molecular structure to medical triazoles.** Molecule alignment studies and docking experiments using a homology model of active site of the Cyp51A

enzyme have identified five triazole fungicides that are very similar to the medical triazoles. These fungicides include propiconazole, tebuconazole, epoxiconazole, difenoconazole and bromuconazole. Bioinformatic analysis showed that the presence of the L98H mutation most hindered the docking of the triazole fungicides with a structure similar to the medical triazoles. Furthermore, the findings were consistent with *in vitro* susceptibility testing, which showed that these compounds were active against wild-type *A. fumigatus*, but inactive against TR_{34}/L98H isolates. Furthermore, the five triazole fungicides were authorised for use in the Netherlands between 1990 and 1996, which preceded the first culture of TR_{34}/L98H from a clinical specimen in 1998 [80].

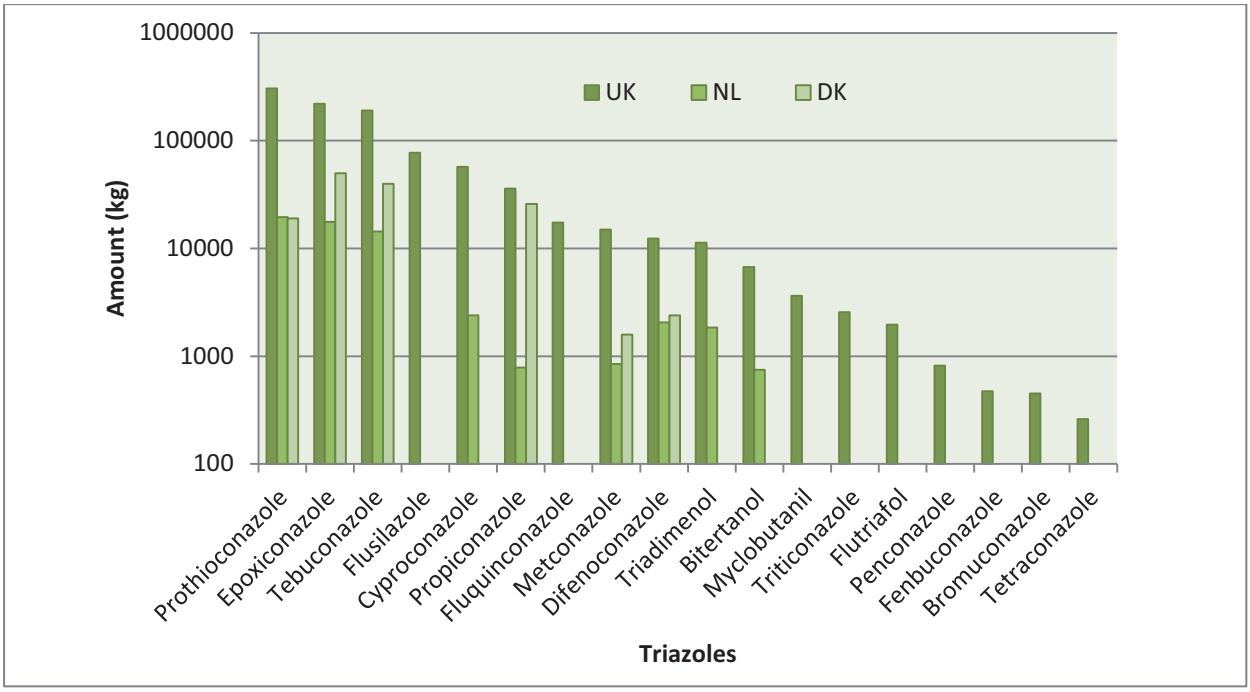
- **Absence of genotypical wild-type isolates related to those with TR_{34}/L98H.** In some patients who have developed azole resistance during azole therapy, isogenic isolates have been recovered with a wild-type phenotype. This indicated that the resistance mechanism developed through the exposure to azoles. However, in patients from whom TR_{34}/L98H isolates were recovered, an identical or strongly related isolate with a wild-type phenotype has never been recovered. In specific patient populations where repeated cultures are available, (e.g. patients with cystic fibrosis) there has repeatedly been no relation to previously cultured wild-type isolates. This observation supports an exogenous source of TR_{34}/L98H.

Use of triazoles and other sterol demethylation inhibitor (DMI) fungicides in agriculture

Since their introduction more than thirty years ago, azoles have played an important role in agriculture and horticulture [81]. Because of their excellent systemic broad-spectrum eradicant and protectant properties, azoles are for many crops (e.g. cereals and soybean) the most important fungicide group to protect from diseases, ensuring yields and preventing fungal contamination of produce, thus ensuring produce quality.

DMI fungicides represent the largest class of fungicides in agriculture and horticulture, with 26.1% of total fungicide sales (USD 8 916 million) globally reported in 2005 [82]. Detailed data on the use of DMI fungicides are not available from all countries. Although the absolute amounts used will differ, the compounds and their purposes are assumed to be similar throughout the EU. As examples of the use of DMI fungicides in agriculture we chose data from the United Kingdom, the Netherlands and Denmark. Figure 1 shows the amounts of individual DMI fungicides used for crop protection in these three countries in 2008.

Figure 1. Use of triazoles in agriculture in the United Kingdom, the Netherlands and Denmark, 2008 (logarithmic scale)



UK: data were collected by the Pesticide Usage Survey Teams at the Food and Environment Research Agency (FERA) and the Scottish Agricultural Science Agency (SASA). Fungicide use by government, industry/forestry and garden/household is not included.
NL. Data were obtained from the Central Bureau of Statistics (Statistics Netherlands). Data on fungicide use by government, industry/forestry and garden/household is included.
DK: Data collected by the Danish Environmental Protection Agency (Miljøstyrelsen) 4/2009.

Most of the listed DMI fungicides are triazoles. The exceptions are the imidazoles (imazalil, prochloraz and triflumizole), the pyridine pyrifenox and the piperazine triforine. Triazoles (e.g. prothioconazole, epoxiconazole, tebuconazole, cyproconazole, metconazole, propiconazole, difenoconazole and flusilazole) and the imidazole prochloraz are most commonly used on arable crops (e.g. wheat, barley, oilseed rape and beans), controlling septoria leaf blotch, rhynchosporium, *Fusarium*, rusts and mildews. These are therefore used over the largest areas and in the highest amounts. Other triazoles (e.g. triadimenol, fluquinconazole, triticonazole, flutriafol, bitertanol) and prochloraz are used as seed treatments for cereal crops to control *Fusarium* seedling blight on wheat, and loose smut and leaf stripe on barley. Prochloraz is also used to sterilise bulbs (e.g. narcissus, tulip and hyacinth), whereas other triazoles such as myclobutanil, penconazole and fenbuconazole are used to control diseases of outdoor bulb/flower crops, vegetables, soft fruit, ornamental crops and orchards (e.g. *Alternaria* fruit rot, *Botrytis* crown rot, apple scab, downy mildew and mildew). Table 4 shows in more detail how different DMI containing products are used in some key crops in the UK.

Table 4. Use of triazole fungicides in the production of selected crops/commodities in the United Kingdom

Crop	Year[a]	Production area (ha)	Key triazole fungicide products[b]	Area treated (ha)[c]	Amount used (kg)	Average spray number[d]	Average dose rate[e]
Beans	2008	118 462	Tebuconazole	65 691	10 222	1.32	0.62
			Cyproconazole/chlorothalonil	24 899	14 938	1.19	0.72
Carrots	2007	15 380	Tebuconazole	11 131	2 328	1.56	0.84
Wheat	2008	2 068 104	Epoxiconazole	1 242 366	75 598	1.46	0.49
			Prothioconazole/tebuconazole	571 313	89 840	1.57	0.54
Dessert apples	2008	5 577	Myclobutanil	22 356	1 309	4.70	0.64
			Penconazole	11 987	386	3.30	0.65
Field roses	2009	324	Myclobutanil	446	20	2.47	volumetric
Herbaceous plants	2009	910	Prochloraz/propiconazole	163	39	4.00	0.39
Hops	2008	1 071	Myclobutanil	4 139	162	4.86	volumetric
Oilseed rape	2008	597 706	Flusilazole/carbendazim	286 508	44 283	1.26	0.52
			Prothioconazole	225 120	22 877	1.24	0.58
			Metconazole	180 784	7 442	1.15	0.57
Outdoor bulbs	2009	4 875	Tebuconazole	6 311	966	1.65	0.61
Strawberries	2006	4 480	Myclobutanil	8 084	634	2.35	0.85
Vines	2006	856	Myclobutanil	1 577	80	2.62	1.02

[a] Year when crops were surveyed by the Food and Environment Research Agency (FERA) and the Scottish Agricultural Science Agency (SASA). Wheat and beans includes winter and spring grown varieties.

[b] Triazoles were applied as solo or in mixtures with other sterol demethylation inhibitors or fungicides with different modes of action.

[c] Area treated is the total number of hectares sprayed.

[d] Average spray number is the average number of applications of the product on the area treated.

[e] Average dose rate is defined as the average proportion of full label rate.

It is clear that the highest amounts of DMI fungicides are used to control diseases in arable crops, grown over large areas and sprayed on average three times in winter (e.g. wheat). The highest selection pressure is on diseases of soft fruit, herbaceous plants, hops and fruit trees where some products are applied up to six times during the season as part of the fungicide programme. Due to the selection pressure created by DMI fungicides, resistance or decreased sensitivity has been reported for key crop/commodity pathogens such as *Mycosphaerella graminicola* (wheat), *Rhynchosporium secalis* (barley), *Botrytis cinerea* (strawberry), *Pyrenopeziza brassicae* (oilseed rape), *Venturia inaequalis* (apple) and *Monilinia fructicola* (stone fruit). In order to control the rate of resistance development, the Fungicide Resistance Action Committee (FRAC), has devised guidelines for the use of DMI fungicides based on the rotational use of fungicides with different modes of action, limiting the number of applications of particular products and recommending use of products at label rates [83].

A recent EU Regulation (1107/2009) 'concerning the placing of plant protection products on the market' and a Directive (2009/128/EC) adopted at the same time on 'establishing a framework for Community action to achieve the sustainable use of pesticides' could have implications on the future registration and use of pesticides, including DMI fungicides, in the EU.

Non-agricultural use of triazole fungicides

The triazoles difenoconazole, tebuconazole and propiconazole are used for the control of key diseases on lawns (*Fusarium* patch, anthracnose and dollar spot) and ornamentals (mildew and rusts) by professionals (greens and golf courses) and amateurs (gardening). Tebuconazole and propiconazole complement each other with regard to efficacy against wood decaying fungi (e.g. *Gloephyllum trabeum* and *Poria* spp.). Together with copper carbonate, these fungicides/biocides are the main components of copper organic wood preservatives used in industry to pressure treat timbers to achieve long-lasting preventive protection of fencing, cladding, plywood, roofing and garden decking. Copper-triazole-based preservatives are widely marketed under the Wolmanized® brand in North America and the Tanalith® brand across Europe. Wood preservatives containing propiconazole and tebuconazole are also available for domestic curative use. Propiconazole for example is registered for use in adhesives, paints, leather, paper and textiles [84] and is available in the UK as the active ingredient in an antifouling agent, biocidal paints and surface biocides.

What happens if we do nothing?

If left unchecked, azole resistance could continue to spread in the environment, especially given the global market in produce. The molecular epidemiology of TR_{34}/L98H indicates that the resistance mechanism developed locally and isolates harbouring this trait subsequently migrated across the borders of European countries [79]. Isolates with the TR_{34}/L98H genetic alteration have also been recovered from Asia (China and India), but it remains unclear whether these are genetically similar to the European isolates.

Another risk is the development of novel resistance mechanisms in the environment as the exposure to azole compounds continues. Such a scenario was recently reported in the Netherlands where a new resistance mechanism, consisting of a 46-base pair tandem repeat and two *cyp51A* substitutions (TR_{46}/Y121F/T289A), has emerged. Within one year, *A. fumigatus* isolates harbouring TR_{46}/Y121F/T289A were recovered from 15 unrelated patients in six different Dutch hospitals. The characteristics of this resistance mechanism resembled that observed for TR_{34}/L98H: a complex resistance mechanism, recovery from azole-naive patients, genotypic clustering apart from TR_{34}/L98H and wild-type isolates, and culture of TR_{46}/Y121F/T289A from the environment. It is anticipated that TR_{46}/Y121F/T289A will migrate in addition to TR_{34}/L98H, thus accelerating the prevalence of azole resistance [85].

Given the high frequencies of allergies and asthma, the aging population, the attendant increase in cancers and their treatment and the expanding indications for transplantation, the numbers of patients at risk of developing aspergillosis looks set to rise relentlessly. The burden of pulmonary disorders is also high and likely to increase. Under these circumstances, acquisition of an azole-resistant strain of *Aspergillus* spp. will likely go unnoticed until the patient fails initial treatment, typically with a triazole antifungal. Hence, the burden of disease may increase further for the individual patient who will require other treatments for longer periods, thus increasing medical costs that are already being stretched even in the better-performing economies. It is also unlikely that new medical antifungals will become available in the near future. Consequently, we will have to make do with the ones we already have. Widespread azole resistance will inevitably mean the loss of one of the three classes of antifungals currently employed to treat aspergillosis. Moreover, none of the echinocandins has been licensed as first-line treatment of the disease which, if azole resistance becomes widespread, will only leave amphotericin B, albeit in different formulations, for patient treatment. Even if echinocandins prove effective, both they and amphotericin B formulations must be administered intravenously several times a week, which usually requires day admission at a healthcare facility. The future will look bleak indeed for the patient and society as a whole should the azole resistance become widespread.

Although the threat is evident, at the moment we can only provide an educated guess as to the extent of the danger involved. Hard facts are required which must be obtained under the right circumstances, as in the Netherlands which has the highest rate of environmental azole-resistant *Aspergillus* spp. It is important to determine the balance between the costs and benefits of azole use in human health on the one hand and non-medical purposes on the other hand. Specifically, the source of azole resistance must be confirmed and the effect of reducing azole fungicide use on resistance must be investigated to ascertain whether it will have a measurable effect. It has been shown that azole-resistant *A. fumigatus* isolates that harbour a *cyp51A*-mediated resistance mechanism have a similar fitness or virulence to wild-type isolates [86]. Both *in vitro* and experimental research indicates that there is no loss of virulence in these azole-resistant isolates. This has also been shown for TR_{34}/L98H. It is clear that TR_{34}/L98H could not have survived in our environment in competition with wild-type isolates if its fitness had been reduced. It is therefore important to investigate what the impact will be if exposure to certain azole fungicides is removed.

This will require funding and concerted efforts being made by all interested parties to share information and expertise.

Recommendations

- Improve epidemiological surveillance through:
  - Routine triazole susceptibility testing for clinical isolates (if antifungal treatment is indicated);
  - Increased and continuous surveillance of triazole resistance in *A. fumigatus* in each EU Member State;
  - Strengthen mycology function at ECDC to monitor disease frequency and triazole resistance, facilitate Member State adoption of recommendations and impact of any public health interventions.
- Develop molecular methods to detect triazole resistance in culture-negative specimens and implement them in laboratory practice.
- Investigate the environmental origin through environmental and laboratory studies, including:
  - Field studies with different triazoles which are putative resistance generators with quantitative and sensitive means of detecting triazole resistance in the environment;
  - Extensive and continued environmental studies;
  - Non-agricultural studies (triazoles are also used extensively by industry, application professionals and amateurs for disease control of lawns/ornamentals and preservation of materials such as wood, coating/paints and plasters);
  - Studies on the reversal of rising resistance rates by restricting certain triazole applications will help to identify appropriate public health measures.

Summary of evidence

The molecular basis of the development of antifungal drug resistance is not well understood and needs to be investigated further to assess the impact and development of this phenomenon. Over the last few years, evidence has been accumulating to indicate an environmental route of resistance development which could have serious implications for the management of infected patients. Both the health and economic consequences of such a development would be considerable, though difficult to quantify as the available data are incomplete for many countries. Importantly, aspergillosis is an environmentally acquired infection. Evidence indicates that azole resistance in *Aspergillus fumigatus* is emerging, not only in the Netherlands but also in other European countries and possibly globally, and that the development of resistance may in part be environmentally driven. Evidence that supports this environmental route of resistance development includes:

- the presence of azole-resistant *Aspergillus* disease in azole-naive patients;
- the dominance of a single and stable resistance mechanism (TR_{34}/L98H);
- the presence of a tandem repeat in the promoter of the target gene *cyp51A*, which is a genomic change not found in any of the *A. fumigatus* isolates that have become resistant through patient therapy, but present in azole-resistant phytopathogenic moulds;
- the recovery of *A. fumigatus* isolates from the environment in Europe which genetically cluster to patient *A. fumigatus* TR_{34}/L98H isolates and are distinct from susceptible (wild-type) *A. fumigatus* isolates;
- the medical azole-resistant *A. fumigatus* isolates are cross-resistant to five triazole fungicides (propiconazole, tebuconazole, epoxiconazole, difenoconazole, and bromucazole) that were introduced for agriculture use between 1990 and 1996, just preceding the first culture of TR_{34}/L98H from a clinical specimen in 1998, and
- molecule alignment and docking of these five triazole fungicides into the active site of the Cyp51A enzyme are identical to that of the medical azoles.

Similarly, there is also evidence that:

- the fitness of the TR_{34}/L98H *A. fumigatus* isolates is not affected by the mutation;
- TR_{34}/L98H associated resistance is generally correlated with multi- or pan-azole resistance;
- patients infected with an azole-resistant *A. fumigatus* isolate are less likely to respond to therapy resulting in an unacceptably high mortality rate approaching 90%;
- the five triazole fungicides (propiconazole, tebuconazole, epoxiconazole, difenoconazole, and bromucazole) are extensively used in the environment for crop protection (e.g. cereals, fruit, vegetables, flowers and ornamentals), control of lawn diseases and preservation of materials all over the EU;
- in addition to the TR_{34}/L98H, comparable resistant mechanisms evolve with other resistant phenotypes due to azole fungicide exposure, thereby extending the problem of the emerging of *A. fumigatus* resistance.

In spite of the data supporting an environmental origin of the multi-azole resistance in *A. fumigatus* due to the TR_{34}/L98H mutation, several uncertainties still exist. Estimates of the prevalence and spread of multi-azole resistance in *A. fumigatus* due to the TR_{34}/L98H mutation are currently based on a small number of studies from a few European countries. Existing data show a strong variation in the frequency and composition of the mutations associated with azole resistance between the centres within and between European countries. It is currently

unclear to what extent these differences are driven by host or environmental factors. Although as shown there is accumulating evidence for an environmental origin of multi-azole resistance in *A. fumigatus*, this link still needs to be proven. Association of certain azole fungicides with the observed resistance mechanism appears likely, yet it is unclear which exposures represent the main drivers for resistance development in *Aspergillus* spp.

In order to understand the epidemiology and origin of azole-resistant *A. fumigatus* in Europe and to encounter and battle this very significant problem, it is essential:

- to obtain good and reliable data from the EU concerning *A. fumigatus* resistance in the patient population and the environment and
- to further identify the cause or the sources of the development of azole resistance in both the clinical setting and the environment.

References

1. EUROSTAT. Yearbook 2011. [Accessed 30th July 2012] Available from: http://epp.eurostat.ec.europa.eu/cache/ITY_OFFPUB/KS-CD-11-001/EN/KS-CD-11-001-EN.PDF.
2. Lass-Florl C, Griff K, Mayr A, Petzer A, Gastl G, Bonatti H, et al. Epidemiology and outcome of infections due to *Aspergillus terreus*: 10-year single centre experience. Br J Haematol. 2005;131(2):201-7.
3. Balajee SA, Kano R, Baddley JW, Moser SA, Marr KA, Alexander BD, et al. Molecular identification of *Aspergillus* species collected for the Transplant-Associated Infection Surveillance Network. J Clin Microbiol. 2009 Oct;47(10):3138-41.
4. Cuenca-Estrella M, Gomez-Lopez A, Mellado E, Monzon A, Buitrago MJ, Rodriguez-Tudela JL. Activity profile *in vitro* of micafungin against Spanish clinical isolates of common and emerging species of yeasts and molds. Antimicrob Agents Chemother. 2009 May;53(5):2192-5.
5. Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, et al. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerg Infect Dis. 2009 Jul;15(7):1068-76.
6. Bueid A, Howard SJ, Moore CB, Richardson MD, Harrison E, Bowyer P, et al. Azole antifungal resistance in *Aspergillus fumigatus*: 2008 and 2009. J Antimicrob Chemother. 2010 Oct;65(10):2116-8.
7. Diaz-Guerra TM, Mellado E, Cuenca-Estrella M, Rodriguez-Tudela JL. A point mutation in the 14alpha-sterol demethylase gene *cyp*51A contributes to itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother. 2003 Mar;47(3):1120-4.
8. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Cuenca-Estrella M, Rodriguez-Tudela JL. Substitutions at methionine 220 in the 14alpha-sterol demethylase (Cyp51A) of *Aspergillus fumigatus* are responsible for resistance *in vitro* to azole antifungal drugs. Antimicrob Agents Chemother. 2004 Jul;48(7):2747-50.
9. Snelders E, van der Lee HA, Kuijpers J, Rijs AJ, Varga J, Samson RA, et al. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. PLoS Med. 2008 Nov 11;5(11):e219.
10. van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, et al. Clinical implications of azole resistance in *Aspergillus fumigatus*, the Netherlands, 2007-2009. Emerg Infect Dis. 2011 Oct;17(10):1846-54.
11. Verweij PE, Howard SJ, Melchers WJ, Denning DW. Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints. Drug Resist Updat. 2009 Dec;12(6):141-7.
12. Mellado E, Garcia-Effron G, Alcazar-Fuoli L, Melchers WJ, Verweij PE, Cuenca-Estrella M, et al. A new *Aspergillus fumigatus* resistance mechanism conferring *in vitro* cross-resistance to azole antifungals involves a combination of *cyp*51A alterations. Antimicrob Agents Chemother. 2007 Jun;51(6):1897-904.
13. Lockhart SR, Frade JP, Etienne KA, Pfaller MA, Diekema DJ, Balajee SA. Azole resistance in *Aspergillus fumigatus* isolates from the ARTEMIS global surveillance study is primarily due to the TR/L98H mutation in the *cyp*51A gene. Antimicrob Agents Chemother. 2011 Sep;55(9):4465-8.
14. Verweij PE, Mellado E, Melchers WJ. Multiple-triazole-resistant aspergillosis. N Engl J Med. 2007 Apr 5;356(14):1481-3.
15. Mortensen KL, Jensen RH, Johansen HK, Skov M, Pressler T, Howard SJ, et al. *Aspergillus* species and other molds in respiratory samples from patients with cystic fibrosis: a laboratory-based study with focus on *Aspergillus fumigatus* azole resistance. J Clin Microbiol. 2011 Jun;49(6):2243-51.
16. Chowdhary A, Kathuria S, Randhawa HS, Gaur SN, Klaassen CH, Meis JF. Isolation of multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR/L98H mutations in the *cyp*51A gene in India. J Antimicrob Chemother. 2012 Feb;67(2):362-6.
17. Morio F, Aubin GG, Danner-Boucher I, Haloun A, Sacchetto E, Garcia-Hermoso D, et al. High prevalence of triazole resistance in *Aspergillus fumigatus*, especially mediated by TR/L98H, in a French cohort of patients with cystic fibrosis. J Antimicrob Chemother. 2012 Aug;67(8):1870-3.
18. Tortorano AM, Venier A, Prigitano A, Esposto MC, Cogliati M. Azole resistant *Aspergillus fumigatus* in the environment in Italy [abstract]. 18th Congress of the International Society for Human and Animal Mycology; 11-15 June 2012; Berlin, Germany; abstr. P745.
19. Hamprecht A, Buchheidt D, Vehreschild J, Cornely O, Spiess B, Plum G, et al. Azole-resistant invasive aspergillosis in a patient with acute myeloid leukaemia in Germany. Euro Surveill. 2012;17(36).
20. Beasley R. The Global Burden of Asthma Report; Global Initiative for Asthma (GINA) 2004 [updated 2004, accessed 3rd March 2010]; Available from: http://www.ginasthma.org/Reports/reports-global-burden-of-asthma.html.
21. Denning DW, Pleuvry A, Cole DC. Global burden of allergic bronchopulmonary aspergillosis with asthma and its complication chronic pulmonary aspergillosis in adults. Submitted for publication.
22. Donnelly SC, McLaughlin H, Bredin CP. Period prevalence of allergic bronchopulmonary mycosis in a regional hospital outpatient population in Ireland 1985-88. Ir J Med Sci. 1991 Sep;160(9):288-90.
23. Eaton T, Garrett J, Milne D, Frankel A, Wells AU. Allergic bronchopulmonary aspergillosis in the asthma clinic. A prospective evaluation of CT in the diagnostic algorithm. Chest. 2000 Jul;118(1):66-72.
24. Al-Mobeireek AF, El-Rab M, Al-Hedaithy SS, Alasali K, Al-Majed S, Joharjy I. Allergic bronchopulmonary mycosis in patients with asthma: period prevalence at a university hospital in Saudi Arabia. Respir Med. 2001 May;95(5):341-7.
25. Benatar SR, Keen GA, Du Toit Naude W. *Aspergillus* hypersensitivity in asthmatics in Cape Town. Clin Allergy. 1980 May;10(3):285-91.
26. Ma YL, Zhang WB, Yu B, Chen YW, Mu S, Cui YL. [Prevalence of allergic bronchopulmonary aspergillosis in Chinese patients with bronchial asthma]. Zhonghua Jie He He Hu Xi Za Zhi. 2011 Dec;34(12):909-13.
27. Koshak EA. Classification of asthma according to revised 2006 GINA: evolution from severity to control. Ann Thorac Med. 2007 Apr;2(2):45-6.
28. Wenzel S. Severe asthma in adults. Am J Respir Crit Care Med. 2005 Jul 15;172(2):149-60.
29. Siroux V, Pin I, Pison C, Kauffmann F. [Severe asthma in the general population: definition and prevalence]. Rev Mal Respir. 2004 Nov;21(5 Pt 1):961-9.
30. Langley SJ, Goldthorpe G, Truman N, Woodcok A, Custovic A. Impaired lung function in asthma is associated with sensitization to airborne moulds. Am J Respir Crit Care Med. 2004;169:A309.
31. Black PN, Udy AA, Brodie SM. Sensitivity to fungal allergens is a risk factor for life-threatening asthma. Allergy. 2000 May;55(5):501-4.
32. O'Driscoll BR, Hopkinson LC, Denning DW. Mold sensitization is common amongst patients with severe asthma requiring multiple hospital admissions. BMC Pulm Med. 2005;5:4.
33. Agarwal R, Noel V, Aggarwal AN, Gupta D, Chakrabarti A. Clinical significance of *Aspergillus* sensitisation in bronchial asthma. Mycoses. 2011 Sep;54(5):e531-8.
34. Stevens DA, Moss RB, Kurup VP, Knutsen AP, Greenberger P, Judson MA, et al. Allergic bronchopulmonary aspergillosis in cystic fibrosis--state of the art: Cystic Fibrosis Foundation Consensus Conference. Clin Infect Dis. 2003 Oct 1;37 Suppl 3:S225-64.

35. Mehta G, Macek M, Jr., Mehta A. Cystic fibrosis across Europe: EuroCareCF analysis of demographic data from 35 countries. J Cyst Fibros. 2010 Dec;9 Suppl 2:S5-S21.
36. Smith NL, Denning DW. Underlying conditions in chronic pulmonary aspergillosis including simple aspergilloma. Eur Respir J. 2011 Apr;37(4):865-72.
37. Denning DW, Pleuvry A, Cole DC. Global burden of chronic pulmonary aspergillosis as a sequel to pulmonary tuberculosis. Bull World Health Organ. 2011 Dec 1;89(12):864-72.
38. Denning DW, Pleuvry A, Cole DC. Global burden of chronic pulmonary aspergillosis complicating sarcoidosis. Eur Respir J. 2012 Jun 27 [Epub ahead of print] Jun 27.
39. Sant M, Allemani C, Tereanu C, De Angelis R, Capocaccia R, Visser O, et al. Incidence of hematologic malignancies in Europe by morphologic subtype: results of the HAEMACARE project. Blood. 2010 Nov 11;116(19):3724-34.
40. Pagano L, Caira M, Candoni A, Offidani M, Fianchi L, Martino B, et al. The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. Haematologica. 2006 Aug;91(8):1068-75.
41. Slobbe L, Polinder S, Doorduijn JK, Lugtenburg PJ, el Barzouhi A, Steyerberg EW, et al. Outcome and medical costs of patients with invasive aspergillosis and acute myelogenous leukemia-myelodysplastic syndrome treated with intensive chemotherapy: an observational study. Clin Infect Dis. 2008 Dec 15;47(12):1507-12.
42. Perkhofer S, Lass-Florl C, Hell M, Russ G, Krause R, Honigl M, et al. The Nationwide Austrian *Aspergillus* Registry: a prospective data collection on epidemiology, therapy and outcome of invasive mould infections in immunocompromised and/or immunosuppressed patients. Int J Antimicrob Agents. 2010 Dec;36(6):531-6.
43. Baldomero H, Gratwohl M, Gratwohl A, Tichelli A, Niederwieser D, Madrigal A, et al. The EBMT activity survey 2009: trends over the past 5 years. Bone Marrow Transplant. 2011 Apr;46(4):485-501.
44. Pagano L, Caira M, Nosari A, Van Lint MT, Candoni A, Offidani M, et al. Fungal infections in recipients of hematopoietic stem cell transplants: results of the SEIFEM B-2004 study--Sorveglianza Epidemiologica Infezioni Fungine Nelle Emopatie Maligne. Clin Infect Dis. 2007 Nov 1;45(9):1161-70.
45. Bulpa P, Dive A, Sibille Y. Invasive pulmonary aspergillosis in patients with chronic obstructive pulmonary disease. Eur Respir J. 2007 Oct;30(4):782-800.
46. Guinea J, Torres-Narbona M, Gijon P, Munoz P, Pozo F, Pelaez T, et al. Pulmonary aspergillosis in patients with chronic obstructive pulmonary disease: incidence, risk factors, and outcome. Clin Microbiol Infect. 2010 Jul;16(7):870-7.
47. NHS. Activity report 2009/10, Transplant activity in the UK. 2010; Available from: http://www.uktransplant.org.uk/ukt/statistics/transplant_activity_report/current_activity_reports/ukt/activity_report_2009_10.pdf.
48. Roels L, Rahmel A. The European experience. Transpl Int. 2011 Apr;24(4):350-67.
49. Pappas PG, Alexander BD, Andes DR, Hadley S, Kauffman CA, Freifeld A, et al. Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Clin Infect Dis. 2010 Apr 15;50(8):1101-11.
50. Wunsch H, Angus DC, Harrison DA, Collange O, Fowler R, Hoste EA, et al. Variation in critical care services across North America and Western Europe. Crit Care Med. 2008 Oct;36(10):2787-93, e1-9.
51. Edbrooke DL, Hibbert CL, Corcoran M. Review for the NHS Executive of Adult Critical Care Services: An International Perspective.1999.
52. Trick WE, Fridkin SK, Edwards JR, Hajjeh RA, Gaynes RP. Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989-1999. Clin Infect Dis. 2002 Sep 1;35(5):627-30.
53. Meersseman W, Lagrou K, Maertens J, Van Wijngaerden E. Invasive aspergillosis in the intensive care unit. Clin Infect Dis. 2007 Jul 15;45(2):205-16.
54. Tortorano AM, Dho G, Prigitano A, Breda G, Grancini A, Emmi V, et al. Invasive fungal infections in the intensive care unit: a multicentre, prospective, observational study in Italy (2006-2008). Mycoses. 2012 Jan;55(1):73-9.
55. Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann JW, et al. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med. 2002 Aug 8;347(6):408-15.
56. Greene RE, Schlamm HT, Oestmann JW, Stark P, Durand C, Lortholary O, et al. Imaging findings in acute invasive pulmonary aspergillosis: clinical significance of the halo sign. Clin Infect Dis. 2007 Feb 1;44(3):373-9.
57. Lortholary O, Gangneux JP, Sitbon K, Lebeau B, de Monbrison F, Le Strat Y, et al. Epidemiological trends in invasive aspergillosis in France: the SAIF network (2005-2007). Clin Microbiol Infect. 2011 Dec;17(12):1882-9.
58. Dannaoui E, Borel E, Monier MF, Piens MA, Picot S, Persat F. Acquired itraconazole resistance in *Aspergillus fumigatus*. J Antimicrob Chemother. 2001 Mar;47(3):333-40.
59. Denning DW, Venkateswarlu K, Oakley KL, Anderson MJ, Manning NJ, Stevens DA, et al. Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother. 1997 Jun;41(6):1364-8.
60. Warn PA, Morrissey G, Morrissey J, Denning DW. Activity of micafungin (FK463) against an itraconazole-resistant strain of *Aspergillus fumigatus* and a strain of *Aspergillus terreus* demonstrating *in vivo* resistance to amphotericin B. J Antimicrob Chemother. 2003 Apr;51(4):913-9.
61. Mavridou E, Bruggemann RJ, Melchers WJ, Verweij PE, Mouton JW. Impact of cyp51A mutations on the pharmacokinetic and pharmacodynamic properties of voriconazole in a murine model of disseminated aspergillosis. Antimicrob Agents Chemother. 2010 Nov;54(11):4758-64.
62. Arendrup MC, Perkhofer S, Howard SJ, Garcia-Effron G, Vishukumar A, Perlin D, et al. Establishing *in vitro-in vivo* correlations for *Aspergillus fumigatus*: the challenge of azoles versus echinocandins. Antimicrob Agents Chemother. 2008 Oct;52(10):3504-11.
63. Mavridou E, Bruggemann RJ, Melchers WJ, Mouton JW, Verweij PE. Efficacy of posaconazole against three clinical *Aspergillus fumigatus* isolates with mutations in the *cyp*51A gene. Antimicrob Agents Chemother. 2010 Feb;54(2):860-5.
64. Arendrup MC, Mavridou E, Mortensen KL, Snelders E, Frimodt-Møller N, Khan H, et al. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. PLoS One. 2010;5(4):e10080.
65. Howard SJ, Lestner JM, Sharp A, Gregson L, Goodwin J, Slater J, et al. Pharmacokinetics and pharmacodynamics of posaconazole for invasive pulmonary aspergillosis: clinical implications for antifungal therapy. J Infect Dis. 2011 May 1;203(9):1324-32.
66. EUCAST. Clinical breakpoints for antifungal agents. 2011 [updated 2011-04-27]; Available from: http://www.eucast.org/clinical_breakpoints/.
67. Seyedmousavi S, Bruggemann RJM, Melchers WJG, Mouton JW, Verweij PE. Efficacy and pharmacodynamic of voriconazole combined with anidulafungin in azole resistant invasive aspergillosis [abstract]. 51st Interscience Conference on Antimicrobial Agents and Chemotherapy; 17-20 September 2011; Chicago (IL), USA; abstr. A2-584.
68. Schwartz S, Ruhnke M, Ribaud P, Corey L, Driscoll T, Cornely OA, et al. Improved outcome in central nervous system aspergillosis, using voriconazole treatment. Blood. 2005 Oct 15;106(8):2641-5.
69. Lin SJ, Schranz J, Teutsch SM. Aspergillosis case-fatality rate: systematic review of the literature. Clin Infect Dis. 2001 Feb 1;32(3):358-66.

70. Patterson TF, Kirkpatrick WR, White M, Hiemenz JW, Wingard JR, Dupont B, et al. Invasive aspergillosis. Disease spectrum, treatment practices, and outcomes. I3 Aspergillus Study Group. Medicine (Baltimore). 2000 Jul;79(4):250-60.
71. van der Linden JWM, Arendrup MC, Verweij PE. Prospective international surveillance of azole resistance in *Aspergillus fumigatus*: SCARE-Network [abstract]. 51st Interscience Conference on Antimicrobial Agents and Chemotherapy; 17-20 September 2011; Chicago (IL), USA; abstr. M-490
72. Denning DW, Park S, Lass-Florl C, Fraczek MG, Kirwan M, Gore R, et al. High-frequency triazole resistance found In nonculturable Aspergillus fumigatus from lungs of patients with chronic fungal disease. Clin Infect Dis. 2011 May;52(9):1123-9.
73. Albarrag AM, Anderson MJ, Howard SJ, Robson GD, Warn PA, Sanglard D, et al. Interrogation of related clinical pan-azole-resistant *Aspergillus fumigatus* strains: G138C, Y431C, and G434C single nucleotide polymorphisms in *cyp*51A, upregulation of *cyp*51A, and integration and activation of transposon *Atf*1 in the *cyp*51A promoter. Antimicrob Agents Chemother. 2011 Nov;55(11):5113-21.
74. Keating MR, Guerrero MA, Daly RC, Walker RC, Davies SF. Transmission of invasive aspergillosis from a subclinically infected donor to three different organ transplant recipients. Chest. 1996 Apr;109(4):1119-24.
75. Pegues DA, Lasker BA, McNeil MM, Hamm PM, Lundal JL, Kubak BM. Cluster of cases of invasive aspergillosis in a transplant intensive care unit: evidence of person-to-person airborne transmission. Clin Infect Dis. 2002 Feb 1;34(3):412-6.
76. Camps SM, van der Linden JW, Li Y, Kuijper EJ, van Dissel JT, Verweij PE, et al. Rapid induction of multiple resistance mechanisms in *Aspergillus fumigatus* during azole therapy: a case study and review of the literature. Antimicrob Agents Chemother. 2012 Jan;56(1):10-6.
77. Snelders E, Huis In 't Veld RA, Rijs AJ, Kema GH, Melchers WJ, Verweij PE. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. Appl Environ Microbiol. 2009 Jun;75(12):4053-7.
78. Mortensen KL, Mellado E, Lass-Florl C, Rodriguez-Tudela JL, Johansen HK, Arendrup MC. Environmental study of azole-resistant *Aspergillus fumigatus* and other Aspergilli in Austria, Denmark, and Spain. Antimicrob Agents Chemother. 2010 Nov;54(11):4545-9.
79. Camps SM, Rijs AJ, Klaassen CH, Meis JF, O'Gorman CM, Dyer PS, et al. Molecular epidemiology of *Aspergillus fumigatus* isolates harboring the TR_{34}/L98H azole resistance mechanism. J Clin Microbiol. 2012 Jun 6 [Epub ahead of print] Jun 6.
80. Snelders E, Camps SM, Karawajczyk A, Schaftenaar G, Kema GH, van der Lee HA, et al. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. PLoS One. 2012;7(3):e31801.
81. Russell PE. A century of fungicide evolution. J Agr Sci. 2005 Feb;143:11-25.
82. Morton V, Staub T. A Short History of Fungicides. 2008 [accessed 14 June 2012]; Available from: http://www.apsnet.org/publications/apsnetfeatures/Pages/Fungicides.aspx.
83. Fungicide Resistance Action Commitee. 2012; Available from: http://www.frac.info/frac/index.htm.
84. Environmental Protection Agency. Registration Eligibility Decision: Propiconazole. July 2006; Available from: http://www.epa.gov/oppsrrd1/REDs/propiconazole_red.pdf.
85. Van der Linden JW, Camps SMT, Kampigna GA, et al. Voriconazole-resistant *Aspergillus fumigatus* with a novel *cyp*51A mutation from clinical and environmental origin in the Netherlands: A descriptive study. Submitted for publication.
86. Mavridou E, Meletiadis J, Jancura P, Abbas S, Arendrup MC, Melchers WJG, et al. *In vitro* growth curves predict *in vivo* virulence of clinical *Aspergillus fumigatus* isolates [abstract]. 51st Interscience Conference on Antimicrobial Agents and Chemotherapy; 17-20 September 2011; Chicago (IL), USA; abstr. M-1536.
87. Denning DW, Pleuvry A, Cole DC. Global burden of allergic bronchopulmonary aspergillosis (ABPA) complicating asthma [abstract]. British Thoracic Society Winter Meeting; 1-3 Dec 2010; London, UK; abstr. P183.

HOW TO OBTAIN EU PUBLICATIONS

Free publications:

- via EU Bookshop (http://bookshop.europa.eu);
- at the European Commission's representations or delegations. You can obtain their contact details on the Internet (http://ec.europa.eu) or by sending a fax to +352 2929-42758.

Priced publications:

- via EU Bookshop (http://bookshop.europa.eu).

Priced subscriptions (e.g. annual series of the Official Journal of the European Union and reports of cases before the Court of Justice of the European Union):

- via one of the sales agents of the Publications Office of the European Union(http://publications.europa.eu/others/agents/index_en.htm).

TQ-32-13-107-EN-C

European Centre for Disease Prevention and Control (ECDC)

Postal address:
ECDC, 171 83 Stockholm, Sweden

Visiting address:
Tomtebodavägen 11A, Solna, Sweden

Phone +46 (0)8 58 60 1000
Fax +46 (0)8 58 60 1001
www.ecdc.europa.eu

An agency of the European Union
www.europa.eu

Publications Office

ISBN 978-92-9193-444-7