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## Emergence of *Aspergillus fumigatus* azole-resistance in azole-naïve COPD patients and their homes

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5 **their homes**  
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9 Running Head: *A. fumigatus* azole-resistance in COPD  
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12  
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3 **Abstract** Azole-resistant *Aspergillus fumigatus* (ARAF) has been reported in COPD patients,  
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5 but has not been specifically assessed so far. Here, we evaluated ARAF prevalence in azole-  
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7 naïve COPD patients and their homes, and ARAF circulation between clinical and  
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9 environmental reservoirs. Sixty respiratory samples from 41 COPD patients with acute  
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11 exacerbation, and environmental samples from 36 of these patient's homes were prospectively  
12  
13 collected between August 2011 and February 2015. *A. fumigatus* was detected in respiratory  
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15 samples from 11/41 patients (26.8%) and in 15/36 domiciles (41.7%). *Cyp51A* sequencing  
16  
17 and selection on itraconazole-medium of clinical (n=68) and environmental (n=48) isolates  
18  
19 yielded ARAF detection in 1/41 COPD patients (2.4%) and 2/36 patient's homes (5.6%). The  
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21 clinical isolate had no CYP51A mutation. Two environmental isolates from two patients  
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23 harbored TR<sub>34</sub>/L98H mutation, and one had an H285Y mutation. Coexistence of different  
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25 *cyp51A* genotypes and/or azole-resistance profiles was detected in 3/8 respiratory and 2/10  
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27 environmental samples with more than one isolate, confirming the need for a systematic  
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29 screening of all clinically relevant isolates. The high prevalence of ARAF in COPD patients  
30  
31 and their homes supports the need for further studies to assess the clinical impact of azole-  
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33 resistance in our region.  
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40 **Keywords:** *Aspergillus fumigatus*, COPD, dwelling, mold environmental exposure, azole-  
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42 resistance, electrostatic dust collector  
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#### 47 **Practical implications**

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49 This paper reports the presence of azole-resistant *Aspergillus fumigatus* (ARAF) isolates in  
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51 azole-naive COPD patients and their homes, and a new CYP51A mutation "H285Y"  
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53 associated with azole resistance, found in an environmental isolate. The high prevalence of  
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55 ARAF in dwellings from COPD patients indicates that domestic mold environmental  
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exposure could be a risk factor for the acquisition of ARAF in susceptible hosts. This risk should be taken into account for the management of patients prone to invasive aspergillosis.

PROOF

## 1 INTRODUCTION

Patients with chronic obstructive pulmonary disease (COPD) are at risk for *Aspergillus* colonization<sup>1,2</sup> and invasive aspergillosis (IA), which has a 1.6-3.9% prevalence,<sup>3,4</sup> and is associated with high mortality.<sup>5</sup> Azole-resistant *Aspergillus fumigatus* (ARAF) has been reported in the UK in four itraconazole-treated COPD patients (three with chronic pulmonary aspergillosis and one with IA<sup>6</sup>), in one patient with IA who had received voriconazole for 2 days in Denmark,<sup>7</sup> and in two azole-naïve patients colonized with TR<sub>46</sub>/Y121F/T289A *A. fumigatus* in the Netherlands.<sup>8</sup> However, although the emergence of ARAF is increasingly studied in hematology IA (1%-15.9% prevalence<sup>9-11</sup>), cystic fibrosis (4.5%-8% prevalence<sup>11-13</sup>) or intensive care units (ICU) patients (4.5% prevalence<sup>10</sup>), the epidemiology and potential clinical impact of *A. fumigatus* azole-resistance in COPD patients has not been specifically assessed so far.

Furthermore, the presence of ARAF isolates has also been increasingly reported in the environment in several European countries, in Asia and in South-America, supporting the role of azole fungicides used in agriculture in the emergence of drug resistance.<sup>14,15</sup> Since most patients acquire *A. fumigatus* from the environment, the emergence and spread of azole-resistant strains in the environment will put more humans at risk. However, only a few studies have combined environmental assessment and clinical data, two of them reporting ARAF isolates (TR<sub>34</sub>/L98H or TR<sub>46</sub>/Y121F/T289A) in the soil around the home of hematology patients with IA due to isolates carrying the same mutation.<sup>16,17</sup> Interestingly, in one of these two studies, ARAF isolates were further found in the patient's home.<sup>17</sup> Another study compared the prevalence and CYP51A mutations of clinical and environmental ARAF in different geographic area of Germany and found a higher prevalence of TR<sub>34</sub>/L98H and TR<sub>46</sub>/Y121F/T289A isolates in the environment than in clinical isolates (4-15% vs. 0-3%),



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3 The study was approved by the local ethics committee (North-West Ethics Committee,  
4 University of Rouen, France, referral number 2010-031) and it was registered at  
5 ClinicalTrials.gov (identifier NCT02318524).  
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## 10 11 **2.2 Mycological analysis of clinical and environmental samples, and** 12 13 **screening for *A. fumigatus* azole-resistant isolates** 14 15 16

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18 Sputum samples were half-diluted with a solution of acetylcysteine (10% in water; Digest-  
19 EUR, Eurobio), incubated for 30 min at 37°C, and 10 µL were cultured on half-diluted  
20 Sabouraud agar medium with 0.5 g/L amikacin (SAB) at 30°C, chromogenic agar medium at  
21 37°C and erythritol agar medium at 24°C for 7 days. For OPW, 10 µL of sample were  
22 cultured similarly without pre-treatment. EDC washing, which was performed no later than 48  
23 h post-sample retrieval, consisted of shaking for 10 min in 20 mL of sterile PBS with 0.1%  
24 Tween 80 in a Stomacher, as previously described<sup>23</sup>, and 100 µL of the collected solution  
25 were cultured on SAB at 30°C, benomyl or DG18 agar media at 24°C for 7 days. *A. fumigatus*  
26 and other molds were identified by macroscopic and microscopic characteristics. When more  
27 than 10 *A. fumigatus* isolates were detected, clinical and environmental samples were further  
28 cultured on Sabouraud agar medium containing 4 mg/L of itraconazole (ITZ) at 37°C for 4  
29 days, for selection of azole-resistant isolates. Otherwise, each *A. fumigatus* clinical and  
30 environmental isolate was screened for detection of azole-resistance by dipping a sterile swab  
31 into a 10<sup>6</sup> conidia/ml suspension and streaking it across the surface of an ITZ medium, which  
32 was incubated at 37°C for 4 days.  
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## 2.3 Phenotypic and genotypic confirmation of *A. fumigatus* identification

*A. fumigatus* identification was confirmed by culture at 50°C for 3 days, and, at the genotypic level, by ITS-rDNA and beta-tubulin sequencing, after DNA extraction using QIAamp® DNA Mini Kit (Qiagen, Courtaboeuf, France), as previously described.<sup>13,24</sup> ITS-rDNA and beta-tubulin sequences were deposited in GenBank database under accession numbers KX788215 to KX788446

## 2.4 *Cyp51A* sequencing

Detection of *cyp51A* alterations was performed for all *A. fumigatus* clinical and environmental isolates by sequencing of the *cyp51A* gene and its promoter. The three following primer pairs were used for amplification of three fragments of 725 bp, 705 bp and 826 bp, respectively:

CYP51AF-1 (5'-TAATCGCAGCACCCTCCAG-3') and CYP51AR-1 (5'-GACATCCTTGWGCTTGCCGTTGAG-3'), CYP51AF-2 (5'-TCTACCTGGGCGTTCAGGG-3') and CYP51AR-2 (5'-CTTCGAGGACTTTTGGCTGTGAG-3'), CYP51AF-3 (5'-AACCCTGTTGATGGCTGGTC-3') and CYP51AR-3 (5'-GCAACAACACTTCAGGGCCA-3').

Amplification reactions were performed in a final volume of 50 µL containing 5 µl template DNA, 10 µl reaction buffer (5X), 1.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 0.4 µM of each primer and 2 units Taq polymerase (GoTaq® DNA polymerase, Promega, France). The PCR cycling protocol was performed in a GenAmp 2700 Engine (Applied Biosystems, Life Technologies, Saint Aubin, France) as follows: one initial denaturation step of 10 min at 95°C, followed by 45 cycles consisting of denaturation for 1 min at 95°C, annealing for 1 min at 55°C and extension for 1 min at 72°C, followed by a final

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3 extension for 15 min at 72°C. *Cyp51A* sequences were deposited in GenBank database under  
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5 accession numbers KX788447 to KX788562  
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## 9 10 **2.5 Antifungal susceptibility testing**

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13 ITZ, voriconazole (VOR), posaconazole (POS) and isavuconazole (IVU) MICs were  
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15 determined for CYP51A mutated isolates and for isolates with positive growth on ITZ  
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17 medium, using the European Committee on Antimicrobial Susceptibility Testing (EUCAST)  
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19 microbroth dilution (E.Def 9.3.1) reference method for susceptibility testing.<sup>25</sup> EUCAST  
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21 criteria were used to define susceptibility and resistance.<sup>26</sup>  
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## 25 26 27 **2.6 Homology modeling of *A. fumigatus* CYP51A**

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30 To assess the azole-CYP51A interactions in mutated isolates and the potential role of  
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32 CYP51A mutations in azole-resistance, we modeled *A. fumigatus* CYP51A structure in  
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34 complex with ITZ. The crystal structure of *A. fumigatus* CYP51B in complex with VOR  
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36 (PDB id 4UYM<sup>27</sup>) was used as the 3D template, as the two proteins share 63.6% sequence  
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38 identity. For that purpose, the 3D template was first modified by docking ITZ instead of  
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40 VOR, applying a 3D-structural alignment with the crystal structure of yeast CYP51 bound to  
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42 ITZ (PDB id 4ZDY), and then 20 models were generated using MODELLER-9v17  
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44 program.<sup>28</sup> Among the models, the one with the lowest energy was selected as the final  
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46 model.  
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### 3 RESULTS

#### 3.1 Characteristics of COPD patients; prevalence of *A. fumigatus* colonization and domestic exposure

The mean age of patients was  $63.8 \pm 9.7$  years old, and M/F sex-ratio was 3.2. *A. fumigatus* colonization was detected in respiratory samples from 11/41 patients (26.8%), with 1 to 20 colonies per sample, which yielded 68 clinical isolates. Culture was positive for all EDCs, where 1 to 159 mold colonies were detected. *A. fumigatus* was present in 15 out of 36 EDCs (1 to 19 colonies), yielding a 41.7% frequency of *A. fumigatus* domestic exposure. These 15 EDCs corresponded to five *A. fumigatus* colonized patients and 10 non-colonized patients, and yielded 41 isolates. Seven supplementary isolates were obtained from air or swab samples.

#### 3.2 Phenotypic and genotypic confirmation of *A. fumigatus* identification

Thermotolerance at 50°C, ITS-rDNA and beta-tubulin sequencing confirmed *A. fumigatus* identification for all isolates. ITS-rDNA mutations were detected in 12 clinical strains from two patients and 13 environmental strains from six patient's homes, whereas only two environmental strains presented beta-tubulin gene mutations, one synonymous and one non-synonymous, all other sequences having 100% identity (Table 1).

#### 3.3 *Cyp51A* mutations in clinical and environmental isolates

*Cyp51A* sequencing yielded five mutation profiles in five clinical and five environmental isolates from six different patients (Table 1). TR<sub>34</sub>/L98H mutation was detected in two

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3 environmental isolates from two patients (n°7 and n°22). Mutations F46Y-M172V-N248T-  
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5 D255E-E427K were detected in two clinical isolates from the same patient (n°24) and in one  
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7 environmental isolate (n°7). Another environmental isolate from patient n°9's EDC presented  
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9 mutations F46Y-M172V-E427K. Mutation A284T was detected in three clinical isolates from  
10  
11 one patient (n°41). Lastly, mutation H285Y was detected in one environmental isolate from  
12  
13 patient n°22.

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16 *Cyp51A* synonymous mutations were detected in 13 isolates which presented G1696A  
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18 mutation, including three environmental isolates from three patients (n°2, 7 and 30) and 10  
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20 clinical isolates from two patients (n°21 and 34). Nine clinical isolates from the same patient  
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22 (n°42) presented the non-synonymous mutation C1562T.

### 23 24 25 26 27 **3.4 Selection on ITZ medium and MIC determination**

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30 Culture was positive on ITZ medium for 7 out of 116 isolates, including the two TR<sub>34</sub>/L98H,  
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32 the H285Y, one clinical isolate from patient n°34 with no CYP51A mutation, and the three  
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34 A284T isolates which had a very weak growth. MIC determination yielded pan-azole  
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36 resistance for the two TR<sub>34</sub>/L98H, the CYP51A WT and the H285Y isolates (for which MICs  
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38 were confirmed by the EUCAST Development Laboratory for fungi, Copenhagen, Denmark),  
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40 but the remaining isolates (three A284T, three F46Y-M172V-N248T-D255E-E427K and one  
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42 F46Y-M172V-E427K) were all ITZ and VOR susceptible, and had a discreet increase in POS  
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44 and IVU MICs (Table 2).  
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### 51 52 **3.5 Prevalence of TR<sub>34</sub>/L98H mutation and azole-resistance**

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54 TR<sub>34</sub>/L98H mutation was detected in 2/48 (4.2%) environmental isolates in two different  
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56 patient's homes, yielding a 5.6% (2/36) prevalence of TR<sub>34</sub>/L98H mutation in the COPD  
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3 patient's domestic environment. When taking into account only *A. fumigatus* positive EDCs,  
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5 TR<sub>34</sub>/L98H mutation was detected in 13.3% (2/15) of patient's homes. Regarding azole-  
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7 resistance, four out of 116 clinical or environmental isolates (including the two TR<sub>34</sub>/L98H  
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9 isolates) were pan-azole resistant, yielding a 3.4% overall prevalence of azole resistance.  
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11 Since one of these isolates was clinical and three were environmental, the prevalence of ITZ  
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13 resistance was 1.5% (1/68) and 6.3% (3/48) in clinical and environmental isolates,  
14  
15 respectively. Azole resistance was detected in the environment of two patients (corresponding  
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17 to two TR<sub>34</sub>/L98H and one H285Y isolates), yielding a 5.6% (2/36) prevalence in patient's  
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19 homes, and in clinical samples from one other patient (corresponding to one WT isolate),  
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21 yielding a 2.4% (1/41) prevalence in patients. When taking into account only *A. fumigatus*  
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23 positive samples, pan-azole resistant isolates were detected in 9.1% (1/11) of patients and  
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25 13.3% (2/15) of patient's homes.  
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### 3.6 Circulation of isolates within and between clinical and environmental reservoirs

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38 Coexistence of F46Y-M172V-N248T-D255E-E427K or A284T and WT azole-sensitive  
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40 isolates in the same sample was found in sputa from patients n°24 and 41, respectively (Table  
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42 1). Mutated and WT isolates also coexisted in EDCs from patients n°7 and 22, where one  
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44 TR<sub>34</sub>/L98H isolate was associated to one F46Y-M172V-N248T-D255E-E427K isolate, and  
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46 one TR<sub>34</sub>/L98H was associated to one H285Y and eight WT isolates, respectively. When  
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48 *cyp51A* genotypes were compared for the five patients who had both clinical and  
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50 environmental isolates, four patients only presented WT isolates in both reservoirs, and one  
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52 patient (n°9) harbored one environmental isolate with F46Y-M172V-N248T-D255E-E427K  
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54 mutations whereas no mutation was detected in the clinical isolate.  
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### 3.7 Geographical distribution of clinical and environmental isolates

Clinical and environmental isolates came from patients living in different parts of the North of France Region, but most were close to Lille (Fig. 1). The two TR<sub>34</sub>/L98H environmental isolates were found in two different cities: Faches-Thumesnil and Marcq-en-Baroeul, which are located in urban areas a few kilometers at the South and North of Lille, respectively. The H285Y isolate was also detected in Faches-Thumesnil, whereas the patient who was colonized with three A284T clinical isolates was living in Marquillies, in a rural area. Isolates with F46Y-M172V-N248T-D255E-E427K and F46Y-M172V-E427K were localized in Lille and Faches-Thumesnil.

### 3.8 Modeling of *A. fumigatus* CYP51A: structural localization of H285

In the 3D model of CYP51A, the H285 residue was a buried residue located at the N-terminal part of the  $\alpha$ -helix I, which is an important part of channel 1, and that pass through the protein structure and interacts in its middle with the heme cofactor (Fig. 2). H285 was then situated quite far from the heme moiety, but along to the entrance of the putative ligand access channel 1.<sup>29</sup> ITZ molecule was, contrariwise, found in the putative channel 2.

## 4 DISCUSSION

The 23.1% prevalence of *A. fumigatus* colonization we found during COPD acute exacerbation is similar to the 16.6%-28% ones that have been reported previously in Spain or in UK.<sup>1,2</sup> Since species identification is rarely done when assessing indoor environments, limited data are available on *A. fumigatus* frequency in dwellings. However, the 41.7%

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3 frequency found in patient's homes in the present study seemed high when compared to some  
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5 other studies that used culture (20%<sup>30</sup>). Since only five out of the 15 *A. fumigatus* positive  
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7 EDCs originated from *A. fumigatus* colonized patient's homes, no relationship between *A.*  
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9 *fumigatus* exposition and colonization could be evidenced. These data were consistent with  
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11 those found in homes from patients with asthma, showing the absence of association between  
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13 *A. fumigatus* isolation from sputum and dustborne levels of *A. fumigatus*.<sup>31</sup> This absence of  
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15 relationship probably results from host individual risk factors for colonization (such as  
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17 corticoid or long course antibiotic treatments), and exposure to other mold sources such as  
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19 food (e.g. pepper, tea) or occupational or leisure activities, or inhalation of airborne mold  
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21 spores when the patient is not at home. In addition, the absence of standard methods for  
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23 measuring indoor mold levels renders difficult the definition of a human dose-response data  
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25 and the interpretation of these data in terms of human health.<sup>32</sup>

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29 The 13.3% prevalence of TR<sub>34</sub>/L98H isolates in COPD patient's homes (when taking  
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31 into account only *A. fumigatus* positive EDCs) was in agreement with environmental origin of  
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33 this mutation, and is consistent with previous data in the Netherlands, Denmark, Germany, or  
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35 India where 12% (6/49), 11% (4/38), 10% (45/455) or 7% (44/630) of soil samples were  
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37 found to contain TR<sub>34</sub>/L98H isolates, respectively.<sup>18,22,33,34</sup> However, contrarily to most other  
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39 studies, which focused on indoor or outdoor samples around hospitals, we report the presence  
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41 of TR<sub>34</sub>/L98H isolates in the domestic environment of patients who are at risk for IA.  
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43 Furthermore, the two homes with TR<sub>34</sub>/L98H isolates were located in urban areas. Altogether,  
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45 these data confirm the presence of ARAF in the homes of patients at risk for IA that was  
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47 recently reported,<sup>17</sup> and further indicate that exposure to environmental ARAF isolates is not  
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49 limited to rural areas where fungicides are used. Since the two patients with TR<sub>34</sub>/L98H  
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51 isolates in their homes were not colonized by *A. fumigatus*, no transmission of TR<sub>34</sub>/L98H  
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53 environmental isolates could be evidenced in our study. However, our data confirms that,  
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3 although most IA with TR<sub>34</sub>/L98H isolates were reported in hematology, transplant or cancer  
4 patients,<sup>8,16,35</sup> COPD patients are also exposed and at risk to be colonized by azole-resistant  
5 isolates and to further develop IA.  
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10 Interestingly, coexistence of different *cyp51A* genotypes and/or azole-resistance  
11 profiles was found in 3/8 (37%) respiratory and 2/10 (20%) environmental samples where  
12 more than one *A. fumigatus* isolate had been detected. The presence of *Aspergillus* sensitive  
13 and resistant isolates has been reported previously in patients with IA.<sup>7,36</sup> The high  
14 frequencies we found indicate that picking a single colony for MIC determination, which is  
15 usually done in clinical practice, is not sufficient to exclude the presence of azole-resistant  
16 isolates, and confirm that MIC determination from all colonies or screening using azole-  
17 supplemented medium should be performed when patients are to be treated.<sup>36</sup> The usefulness  
18 of this method was confirmed in our study where two ITZ-resistant isolates were obtained  
19 after direct sowing of one EDC and one sputum, which contained more than 10 colonies on  
20 ITZ medium.  
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34 Other CYP51A mutations included A284T, which was reported to be associated with  
35 decreased sensitivity to ITZ, VOR and POS.<sup>37</sup> However, only POS and IVU MICs were  
36 discreetly increased in our study. Interestingly, a new mutation H285Y was also identified in  
37 one environmental isolate, and was associated with pan-azole resistance, IVU MIC being  
38 correlated with VOR MIC, as previously described for ARAF isolates with other CYP51A  
39 mutations.<sup>38</sup> Modeling the CYP51A indicated that the buried residue in position 285 was  
40 situated at the entrance of the ligand access channel 1. Although the mutation of a buried  
41 residue generally induces largest structural rearrangements, no large structural alteration of  
42 the channel 1 was found to be caused by the H285Y mutation. Furthermore, since ITZ was  
43 proposed to enter via another channel, the impact of H285Y modification on the ITZ  
44 susceptibility appears difficult to predict. Further investigations are required to precise the  
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3 functional effect of this mutation, which, interestingly, is localized near the A284T and  
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5 T289A mutations. However, contrarily to H285Y, T289A is not associated with decreased  
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7 azole sensitivity when not combined with TR<sub>46</sub>/Y121F.<sup>39</sup> Surprisingly, although  
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9 TR<sub>46</sub>/Y121F/T289A isolates have been reported in Belgium,<sup>40</sup> which is only at a few  
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11 kilometers from Lille, or in other areas in France,<sup>41</sup> none was found in our study. However, a  
12  
13 potential caveat is that, when more than *A. fumigatus* 10 isolates had been detected, we  
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15 performed a direct screening from samples using ITZ agar only. The TR<sub>46</sub>/Y121F/T289A  
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17 genotype induces high level VOR and IVU resistance but variable if any ITZ MIC elevation,  
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19 and a recent study showed that for reliable detection of this genotype screening using VOR 1  
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21 mg/L agar was necessary.<sup>42</sup> Hence, although *cyp51A* was sequenced for all the *A. fumigatus*  
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23 isolates in our study, we cannot rule out that TR<sub>46</sub>/Y121F/T289A isolates may have been  
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25 missed by our screening procedure in samples containing more than 10 isolates.  
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30 Altogether, one out of 11 of our “azole-naïve” *A. fumigatus* colonized patients  
31  
32 harbored ARAF, yielding a 9.1% prevalence. Regarding the low number of *A. fumigatus*  
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34 colonized patients in our study, this frequency was quite similar to the ones that were reported  
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36 in some studies in Belgium or in France in different situations or populations (such as  
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38 hematology or ICU patients with IA, or cystic fibrosis patients).<sup>9-13</sup>  
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41 In conclusion, the presence of ARAF in indoor environments of patients at risk for  
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43 aspergillosis indicates that home environment should be considered as a potential source of  
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45 exposure to azole resistant isolates in susceptible hosts, such as patients with chronic  
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47 respiratory diseases, or immunocompromised patients. Furthermore, coexistence of *A.*  
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49 *fumigatus* azole sensitive and resistant isolates in clinical and environmental samples support  
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51 the need for a detection of azole-resistant isolates on all *A. fumigatus* colonies in routine  
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53 practice. Our data confirm that using azole containing agar medium can be used either from  
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55 colonies, or directly from samples. Lastly, the high prevalence of ARAF in COPD patients  
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3 and their homes strongly supports the need for further studies to assess the clinical impact of  
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5 ARAF in patients with *Aspergillus* diseases, and for potential re-evaluation concerning the  
6  
7 current choices of antifungal therapy in our region.  
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## 50 51 52 **CONFLICT OF INTEREST**

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PROOF

**TABLE 1.** Characteristics of *A. fumigatus* clinical and environmental isolates: origin, *cyp51A*, *beta-tubulin*, *ITS-rDNA* sequencing, and growth on ITZ medium.

Pan-azole resistant isolates are highlighted in grey. Synonymous mutations are in italics. OPW=Oropharyngeal wash, EDC=Electrostatic Dust Collector

	Patient number	Origin of isolates		Number of isolates	<i>cyp51A</i> sequencing			Mutated $\beta$ - <i>tubulin</i> isolates	Mutated <i>ITS-rDNA</i> isolates	
					WT	Non synonymous mutation	Synonymous mutation			
<i>A. fumigatus</i> colonized patients, no detection in the patient's home	10	Clinical	Sputum	10	10					
	21		Sputum	5	5					
	24		Sputum	6	4	2 F46Y, M172V, N248T, D255E, E427K			2 C11G	
	34		Sputum	12	11+1*					
	41		Sputum	8	5	3 A284T				
	42		Sputum	9			9 C1562T		9 T91C	
			OPW	1	1			1 T91C		
<i>A. fumigatus</i> colonized patients & detection in the patient's home	2	Clinical	Sputum	3	3					
			Environment	Air	1	1				1 T91C
				Swab	1	1				1 T91C
	9	Environment	EDC	4	4			1 V48I	1 T91C	
			Clinical	Sputum	1	1				
	18	Environment	EDC	1	1	1 F46Y, M172V, E427K		1 G60A		
			Clinical	Sputum	1	1				
	19	Environment	EDC	4	4					
Clinical			OPW	1	1					
39	Environment	EDC	4	4						
		Clinical	OPW	11	11					
			EDC	3	3				2 T91C	
<i>A. fumigatus</i> detection in the patient's home, no detection in clinical samples	1	Environment	Air	3	3					
			EDC	2	2				2 T91C	
			Air	1	1					
	7		EDC	2			1 TR34/L98H 1 F46Y, M172V, N248T, D255E, E427K			
				Swab	1	1				1 T91C
	12		EDC	1	1					
	22		EDC	12	10	1 TR34/L98H* 1 H285Y			4 T91C	
	27		EDC	1	1					
	29		EDC	1	1				1 T91C	
	30		EDC	2	2					
31	EDC	1	1							
40	EDC	3	3							
<b>TOTAL</b>				<b>116</b>	<b>97</b>	<b>10</b>	<b>9</b>	<b>2</b>	<b>25</b>	

**TABLE 2.** MIC determination for *A. fumigatus* isolates with positive growth on ITZ medium and/or CYP51A mutation.

The following EUCAST criteria were used to define susceptibility and resistance: an ITZ or VOR MIC of  $\leq 1$  mg/L was considered susceptible, a MIC of 2 mg/L intermediate and of  $> 2$  mg/L resistant; a POS MIC of  $\leq 0.12$  mg/L was considered susceptible, a MIC of 0.25 mg/L was considered intermediate and of  $> 0.25$  mg/L resistant; an IVU MIC of  $\leq 1$  mg/L was considered susceptible, a MIC of  $> 1$  mg/L was considered resistant.<sup>26</sup> Pan-azole resistant isolates are highlighted in grey.

CYP51A mutations	MIC (mg/L)			
	ITZ	VOR	POS	IVU
<b>TR34/L98H (n=2)</b>	>8	4	1-2	8-16
<b>H285Y (n=1)</b>	>16	4	0.5	4
<b>WT (n=1)</b>	>16	4	1	>16
<b>A284T (n=3)</b>	0.25-0.5	0.25-1	0.5	2
<b>F46Y, M172V, N248T, D255E, E427K (n=3)</b>	1	1	0.5	1-2
<b>F46Y, M172V, E427K (n=1)</b>	0.5	1	0.25	1



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3 **FIGURE 2.** Ribbon illustrations of the *A. fumigatus* CYP51A 3D model. Helices/loop and  
4 strands are shown in corral and cyan, respectively. The helix I containing the H285 residue, is  
5 colored in orange. The structure is showed in two different views related by a 90-degree  
6 rotation. The protein surface is displayed in transparency. The heme cofactor (yellow  
7 carbons), ITZ (green carbons) and residue H285 (magenta carbons) are depicted as sticks and  
8 labeled in the right view, as well as the N- and C-terminal regions. The two putative ligand  
9 access channels<sup>29</sup> are labeled and indicated by arrow. Oxygen, nitrogen and iron are colored  
10 in red, blue and orange, respectively. The images were produced using the PyMOL Molecular  
11 Graphics System (Version 1.7, Schrödinger, LLC).  
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