Aspergillus colonisation and antifungal immunity in cystic fibrosis patients.

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<td>Warris, Adilia; MRC Centre for Medical Mycology at the University of Aberdeen Bercusson, Amelia; National Heart and Lung Institute, Imperial College London Armstrong-James, Darius; National Heart and Lung Institute, Imperial College London</td>
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<tr>
<td>Keyword:</td>
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<td>Cystic fibrosis (CF), caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, is the most common inherited life-limiting disease in North European people affecting 90,000 people worldwide. Progressive lung damage caused by recurrent infection and chronic airway inflammation is the major determinant of survival with a median age at death of 29 years. Approximately 60% of CF patients are infected with Aspergillus fumigatus, a ubiquitous environmental fungus, and its presence has been associated with accelerated lung function decline. Half of the patients infected with Aspergillus are &lt;18 years of age. Yet, time of acquisition of this fungus and determinants of CF-related Aspergillus disease severity and progression are not known. CFTR expression has been demonstrated in cells of the innate and adaptive immune system and has shown to be critical for normal function. Research delineating the role of CFTR-deficient phagocytes in Aspergillus persistence and infection in the CF lung, has only recently received attention. In this concise review we aim to present the current understanding with respect to when people with CF acquire infection with A. fumigatus and antifungal immune responses by CF immune cells.</td>
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Aspergillus colonisation and antifungal immunity in cystic fibrosis

patients.

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Abstract

Cystic fibrosis (CF), caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, is the most common inherited life-limiting disease in North European people affecting 90,000 people worldwide. Progressive lung damage caused by recurrent infection and chronic airway inflammation is the major determinant of survival with a median age at death of 29 years. Approximately 60% of CF patients are infected with *Aspergillus fumigatus*, a ubiquitous environmental fungus, and its presence has been associated with accelerated lung function decline. Half of the patients infected with *Aspergillus* are <18 years of age. Yet, time of acquisition of this fungus and determinants of CF-related *Aspergillus* disease severity and progression are not known.

CFTR expression has been demonstrated in cells of the innate and adaptive immune system and has shown to be critical for normal function. Research delineating the role of CFTR-deficient phagocytes in *Aspergillus* persistence and infection in the CF lung, has only recently received attention. In this concise review we aim to present the current understanding with respect to when people with CF acquire infection with *A. fumigatus* and antifungal immune responses by CF immune cells.
Introduction

In cystic fibrosis (CF), 90% of morbidity and mortality is a consequence of chronic suppurative lung disease. Aggressive targeted and chronic treatment of airway infection is the mainstay of clinical management, reducing lung function decline and improving survival. The introduction of CFTR modulators into clinical practice reduces (but does not abolish) pulmonary exacerbations and rate of lung function decline, and will not reverse existing lung damage (bronchiectasis). Even with new treatment modalities targeting CFTR dysfunction, pulmonary infections will continue to remain the major prognostic problem in CF. Research and treatment has conventionally focussed on the role of bacterial pathogens with little attention being paid to the role of fungal species.

Aspergillus fumigatus is a ubiquitous fungus: inhalation of Aspergillus spores and their airway deposition is fact of everyday life. Susceptibility to Aspergillus-related lung disease in CF is reflected by clinical phenotypes ranging from persistent Aspergillus infection and bronchitis to allergic and airway invasive aspergillosis. About 60% of CF patients are infected with A. fumigatus and this has been associated with accelerated lung function decline. Little attention has been paid to the role of A. fumigatus in the pathogenesis of non-ABPA (allergic bronchopulmonary aspergillosis) Aspergillus lung disease in CF, despite its’ frequent recovery in respiratory samples.

In vitro studies have demonstrated that CF phagocytes display reduced killing activity against typical CF bacterial pathogens such as Pseudomonas aeruginosa and Burkholderia cepacia. Others have reported defects in a number of host immune mechanisms including the release of antimicrobial peptides, intracellular alkalization, diminished production of hypochlorous acid, and increased release of pro-inflammatory cytokines. Only a few studies can be found in the literature addressing innate antifungal immune
responses by CFTR-defective phagocytes and epithelial cells against *A. fumigatus*.\textsuperscript{15-17} In addition, 2 studies have addressed the persistent inflammation evoked by *A. fumigatus* (non-allergic) infection in an experimental CF murine model.\textsuperscript{18,19} This concise review summarizes our current understanding addressing the highly relevant questions of when people with CF acquire infection with *A. fumigatus* and why the absence of a functional CFTR protein in immune cells renders them susceptible to develop *Aspergillus* airway disease.

**Aspergillus colonisation**

The isolation of *Aspergillus* species, in particular *A. fumigatus*, in respiratory secretions of patients with CF is a common occurrence.\textsuperscript{20,21} *Aspergillus fumigatus* is a ubiquitous fungus and its conidia are dispersed in the air we breathe. In patients with CF, the impaired mucociliary clearance and airways being filled with thick mucus, the inhaled *Aspergillus* conidia are easily trapped. Numerous studies have reported a high variety in prevalence rates (ranging from 3.2% to 56.7%) and this most likely reflects the differences in culture techniques, the frequency of sampling, the interpretation of the culture results and if fungal cultures are performed as routine practice in clinical management. Authors have aimed to differentiate between transient colonisation (≤ 1/yr) and persistent or chronic (≥2/0.5-1yr) colonisation, although no consensus exists for those definitions, and some studies have used even stricter definitions. Table 1 provides an overview of studies in which either results of fungal cultures were reported as a secondary outcome or in which fungal colonisation was the primary focus of the study.\textsuperscript{6,22-57} Colonisation with *Aspergillus* species may be an apparently isolated finding, but may also be seen in the context of fungal sensitization,
ABPA or *Aspergillus* bronchitis. The majority of the studies summarized in table 1 lack information on this important aspect, and make it difficult to assess *Aspergillus* colonisation on its own as being harmful in terms of CF lung disease progression. To address this issue, a novel diagnostic classification system of *Aspergillus* infection and disease has been proposed and was consequently used to provide estimates on the prevalence of the different phenotypes of aspergillosis in CF using national and international CF registry data.\(^7,58\) As this novel classification system was tested in a cohort of adult patients, it remains to be seen if these frequencies can be used on the total CF population as almost 50% of CF patients are \(\leq\) 18 yrs of age. The estimated prevalences reported did not include patients colonized with *Aspergillus* in the absence of fungal sensitisation, ABPA or *Aspergillus* bronchitis.

Galactomannan and *Aspergillus* PCR on sputum samples have been proposed to improve the detection of *A. fumigatus* and *Aspergillus* disease in CF.\(^58\) Unfortunately, no definition was proposed how those 2 markers should be included to define persistent colonisation.

Most colonisation studies have focussed on *A. fumigatus*,\(^23-25,27,28,33-36,38,39,42,43,45-49,51-55,57\) being the most prevalent *Aspergillus* species encounters in human colonisation and disease, while others have reported *Aspergillus* colonisation without defining the actual species.\(^6,22,26,29,30,32,37,40,41,50,56,59\) Studies reporting on the relative number of *A. fumigatus* versus non-*fumigatus* *Aspergillus* species have shown that between 36% and 58% of *Aspergillus* colonisation in patients with CF is caused by *A. fumigatus*.\(^38,40-42,46\) Jubin et al showed that mixed *Aspergillus* species colonisation may occur in up to 33% of the patients.\(^41\)

Although *Aspergillus* infection and disease in CF is considered to become a problem during adolescence and adulthood, epidemiological studies about when patients with CF acquire *Aspergillus* colonisation are scarce. Epidemiological studies conducted in France, Australia
and in Greece, reported a mean age of the patients at date of first isolation of *A. fumigatus* to be 12.9 yrs, 9 yrs and 13 yrs, respectively. Two more recent studies, reported an age of acquisition being 16.4 yrs (median, range 12.2-22.0 yrs) in the US, 13.5 yrs (mean, SD +/- 4yrs) in France, and 9.0 yrs (median, range 3.1-16.2 yrs) in the UK. Two other studies have broken down the prevalence of *A. fumigatus* colonisation into different age categories showing colonisation rates of 16.4% up to 28% in children < 12 yrs of age. By differentiating transient (≤ 1/yr) from persistent (≥2/yr) colonisation, Saunders et al showed that children < 12 yrs of age were only transiently colonized in a minority of the cases (21%), with the majority of children ≥ 12 yrs (59%) being persistently colonised with *Aspergillus*. Cultures from BAL-fluid showed a significantly higher yield of *A. fumigatus* compared to sputum samples taken in the same period. Valuable data were obtained from an Australian study in which infants, diagnosed with CF after newborn screening, underwent bronchoalveolar lavage (BAL) at the age of 3 mo, 1 yr and 2 yrs. *Aspergillus* was cultured from BAL-fluid of 7 out of 56 infants (12.5%). The difficulties in obtaining sputum samples from infants and young children (< 8 yrs) most likely leads to an underestimation of the prevalence of *Aspergillus* colonisation and infection in this age group, and precludes any conclusion about when patients with CF acquire *Aspergillus* infection.

**Aspergillus colonisation and lung disease**

Several studies have investigated the role of *Aspergillus* colonisation on lung function in CF with conflicting results. Several studies have reported either more pulmonary exacerbations requiring hospitalizations and/or significant lower lung function parameters and/or a steeper lung function decline in *Aspergillus* colonized patients. However, other
studies do not support an independent effect of Aspergillus colonisation on lung function.\textsuperscript{29,32,37,41,43,54,56}

McMahon showed that Aspergillus colonisation, defined as at least two sputum cultures positive for Aspergillus spp. at least four weeks apart in the year prior to study inclusion, resulted in more severe and significant bronchiectasis scored on HRCT-chest.\textsuperscript{60}

Noni et al showed that paediatric patients (n=121, mean age 14 yrs) with chronic Aspergillus colonisation (≥2 positive sputum cultures/yr) had a lower FEV1 at baseline and a faster deterioration of their lung function in the 7 yrs after baseline.\textsuperscript{49} In a large retrospective study using registry data from over 16,000 CF patients, lower FEV1 percent predicted was not a predictor of development of persistent Aspergillus colonisation.\textsuperscript{56}

In a systematic review by Harun et al \textsuperscript{61}, which included studies describing lung function progression over age in CF patients, only 2 out of 39 publications (between 1990-2015) recognized Aspergillus colonisation or disease as a possible risk factor influencing lung function decline.\textsuperscript{5,62} This reflects that Aspergillus infection and disease has not been recognized as playing a major role in the lung function decline in patients with CF, resulting in the absence of systematic approaches to monitor Aspergillus infections and disease longitudinally in CF patients from an early age onwards. A recent survey among paediatric and adult CF consultants in the UK showed that one third of the respondents considered Aspergillus colonisation to be potentially harmful and would therefore treat this condition.\textsuperscript{63}

**Innate fungal immunity**

CFTR-defective epithelial cells
Direct experimental evidence of the interaction between CF epithelial cells and *Aspergillus* conidia is limited. However, there is evidence for the contribution of healthy epithelial cells to the innate immune response to *A. fumigatus* and for impairments in epithelial cell function in CF, from which insights can be gained. CFTR protein is highly expressed in lung epithelial cells and its loss leads to reduced airway surface hydration and impaired mucociliary transport. This is significant as *Aspergillus* conidia are regularly inhaled into the airways and the mucociliary escalator is a key mechanism for preventing colonisation.

Pentraxin 3 is a soluble pattern recognition receptor that is secreted by epithelial cells in response to *A. fumigatus* and is important for *Aspergillus* clearance. Pentraxin 3 levels have been found to be reduced in sputum from CF patients.

Although not the primary phagocytes of the airway, epithelial cells have been shown in-vitro to phagocytose conidia. Adhesion and internalisation of conidia has been shown to be impaired in CF bronchial epithelial cells. In the same paper authors also demonstrated increased baseline and *Aspergillus*-induced apoptosis, reduced chemokine secretion and impaired killing by CF epithelial cells in-vitro. In an in-vivo murine model, they found impaired clearance of *Aspergillus* conidia and evidence of epithelial necrosis and fibrin deposition in CFTR-deficient but not wild-type mouse airways. Ceramide has been shown to accumulate in CF bronchial and alveolar epithelial cells in mice and humans. In *A. fumigatus* infection, ceramide mediates inflammation and interferes with epithelial killing of the fungus. Inhibition of de-novo ceramide synthesis reduced inflammatory cytokine release, granulocyte infiltration and fungal colonisation in a murine model of *A. fumigatus* infection.

CFTR-defective neutrophils
Human neutrophils express the CFTR protein in phagosomes and in the membranes of secretory vesicles and has shown to play a critical role in regulating antimicrobial neutrophil activities.\textsuperscript{70-72} Upregulating of Toll-like receptor (TLR) 5 on human CF airway neutrophils have been described and its signalling results in excessive cytokine production after stimulation with \textit{P. aeruginosa}.\textsuperscript{73,74} A small number of functional studies implicate that the absence of a functional CFTR-protein in phagocytes leads to inadequate intraphagosomal chloride transport and less production of hypochlorous acid (HOCl) resulting in a diminished killing of \textit{P. aeruginosa}.\textsuperscript{9} A study by Pohl et al. showed that impaired CFTR function in neutrophils leads to decreased release of secondary and tertiary granules due to altered ion homeostasis that is corrected by CFTR potentiator therapy.\textsuperscript{75}

We have recently shown that although human CF neutrophils are capable of efficiently phagocytose and kill \textit{A. fumigatus}, this is at a cost of excessive reactive oxygen species (ROS).\textsuperscript{17} Previous studies investigating neutrophil ROS production in vitro have shown contrasting results, with ROS production being either increased, decreased or normal in CF neutrophils.\textsuperscript{76-78} A possible reason for this inconsistency is that this response is stimulus specific as a range of microbial and non-microbial stimuli were used in these studies. The excessive amount of ROS induced by \textit{A. fumigatus} in CF neutrophils is significantly correlated to disease severity in terms of clinical exacerbations and lung function [Brunel 2018].\textsuperscript{17} Our data suggest that the hyper inflammatory response by CF neutrophils upon exposure to \textit{A. fumigatus} is likely to contribute to progressive lung disease.\textsuperscript{17} Increased NLRP3 expression in murine cftr-/- neutrophils was demonstrated, while pNLRC4 expression was inherently lower.\textsuperscript{19} These combined defects were shown to result in increased neutrophil recruitment and IL-1\textbeta release during \textit{Aspergillus} infection in CF mice.\textsuperscript{19}
CFTR-defective macrophages

Alveolar macrophages play a key role in controlling pulmonary fungal infections both through direct killing of phagocytosed pathogens and by regulating the inflammatory response generated by inhaled fungal pathogens. There is evidence from work with bacteria that macrophage antimicrobial functions are impaired in CF but there is little experimental data looking at macrophage interactions with fungi in CF.

CFTR expression has been demonstrated in human monocyte-derived macrophages and murine and human alveolar macrophages. These authors have reported impaired macrophage killing of *P. aeruginosa* and have proposed impaired phagosomal acidification as the mechanism underlying this defect. However, others have challenged this hypothesis. Impaired autophagy has also been reported in CF macrophages and may contribute to the killing defect towards *B. cenocepacia* as observed in one study. A similar autophagy defect has been reported in lung macrophages isolated from *A. fumigatus* infected CFTR-deficient mice. Recently, correction of an anti-*Pseudomonal* killing defect by treatment of CF macrophages with the CFTR corrector Lumacaftor (VX-809) was reported.

Alveolar macrophages are the primary phagocytes of the airway. Failure to kill phagocytosed conidia will result in germination and fungal escape out of the cell. The hyphal form of *Aspergillus* releases proteases and gliotoxins, causing further damage to the host airway.

As well as failing to kill pathogens effectively, CF macrophages have also been observed to generate hyper-inflammatory responses to infectious stimuli. *B. cenocepacia* induced greater inflammatory cytokine release and inflammatory cell death in monocyte-derived macrophages from CF patients versus healthy controls. Inflammatory cytokine release by CF murine and human macrophages stimulated with LPS was increased versus wild type
and healthy controls. In a murine CF model of *A. fumigatus* infection, Ianitti et al observed increased activation of the NLRP3 inflammasome in CF lung macrophages versus wild type controls.

**CFTR-defective T-cells**

The role of T cells in immunity to pulmonary aspergillosis is well recognised with Th1, Th2, Th17, Th9 and cytotoxic T cells all playing a role. Th1 CD4+ T cells enable inflammation and fungal clearance, whilst Th17 cells are important for neutrophil recruitment, Th2 cells for allergic inflammation and Th9 responses play a role in fibrosis. Furthermore, a role for T regulatory (Treg) cells has been described in regulation of innate and adaptive responses. CF lymphocyte unresponsiveness to bacterial pathogens has been long established, with a tendency for a pro-allergic Th2 response and increased tendency to develop allergic *Aspergillus* lung disease in patients with CF. This is consistent with CF murine models where *A. fumigatus* challenge leads to a shift to IgE, IL-4 and IL-13 production. Furthermore, T helper cells from CF patients have lower levels of interferon-γ production and increased IL-10 production. It has been hypothesized that this is a consequence of increased calcium flux across the T cell membrane. Calcium flux has been shown to be regulated by CFTR, and is crucial for T cell activation. Murine studies have specifically demonstrated that T cell receptor stimulation led to increased calcium entry in CFTR-deficient T cells, and increased translocation of the calcineurin-dependent transcription factor NFAT. Thus, excessive calcium flux as a direct consequence of CFTR dysfunction in T cells may be a major contributor to dysregulated immune responses in CF.

Recent studies showed that CFTR−/− mice have increased innate lymphoid cell IL-9 production in response to *A. fumigatus* challenge, which is linked to expansion of Th9 T cells.
neutralisation led to an improvement in lung immunopathology and fibrosis. Further studies in the murine model of pulmonary aspergillosis showed that CFTR dysfunction led to defective indoleamine 2,3-dioxygenase activity, leading to alterations in tryptophan metabolism with a subsequent imbalance of the Th17/Treg axis and excessive lung inflammation.\textsuperscript{18} Therapeutic modulation of this pathway to enhance indoleamine 2,3-dioxygenase enabled resolution of excessive inflammation.\textsuperscript{18} Clinical studies have also shown that the co-stimulatory molecule, OX40 ligand, is critical for driving CD4+ Th2 responses in CF patients with ABPA.\textsuperscript{95} This correlated with low vitamin D levels in serum. Notably ex vivo therapy with vitamin D led to reduced OX40 ligand expression and reduced Th2 responses.\textsuperscript{95} These studies indicate that a better understanding of the hyper-inflammatory T cell responses in CF may open up new immunotherapeutic avenues for the treatment of fungal-driven lung disease.

**Summary and Future Directions**

Aspergillus colonisation is commonly observed in patients with CF, with *A. fumigatus* most frequently encountered, and can manifest in clinically recognized CF-related Aspergillus diseases as ABPA and fungal sensitization. Persistent Aspergillus colonisation or Aspergillus bronchitis are not well defined and should receive more attention. Only incomplete data exists with respect to *A. fumigatus* colonisation dynamics and time of acquisition. In addition, the impact of persistent Aspergillus colonisation (infection) on lung function decline in CF has not been studied sufficiently. A systematic approach in which standardized fungal diagnostic measurements are applied in a longitudinal way to a large cohort of patients with CF, is urgently needed to assess the impact of persistent Aspergillus
colonisation. The findings of such investigations will inform clinical decision making with respect to the need of therapeutic interventions.

Treatment of persistent *Aspergillus* colonisation in the absence or presence of clinical symptoms of pulmonary exacerbation and/or a decline in lung function with antifungals has not been properly assessed. The only study which aimed to target persistent *Aspergillus* infection in patients with CF was flawed by largely underdetectable serum itraconazole concentrations. Well-known adverse effects of long term azole antifungal therapies, including the rapid emerging triazole antifungal resistance, toxicity and interaction with CFTR modulators, will challenge the use of azole antifungals for persistent *Aspergillus* colonisation and infection.

Enhanced mechanistic insights in the impairments in innate antifungal immune mechanisms is warranted to identify new targets for non-allergic *Aspergillus* infections in CF patients. Currently, the observed *Aspergillus*-induced inflammatory responses by innate immune cells in both in vitro, ex vivo and experimental models of infection, seem to be a potential target to limit the pathological consequences of CF-related *Aspergillus* infection. A range of anti-inflammatories are being developed for the general treatment of CF-related airway disease. It will be crucial to understand the impact both on reducing hyper-inflammatory responses as well as whether there is an effect on direct antifungal killing mechanisms. The CFTR modulators are the first causative treatment options for CF patients and have achieved significant improvement in lung function and quality of life. One study showed a clear reduction in fungal colonisation in the CF lung - an effect that may be associated with the mitigation of the impaired innate immune mechanisms observed in CF immune cells. In *vitro* work, which showed that a defective CFTR is associated with impaired degranulation of
antimicrobial proteins in neutrophils, has demonstrated that ivacaftor is able to correct
degranulation and to increase bacterial killing by activation of Rab27a. Another example of
a small molecule based therapy targeting both the primary defect in CF as well as the
aberrant inflammation and immune response, is thymosin α1. Thymosin α1 is a natural
occurring polypeptide, used as an immunomodulator in viral infections, malignancies and
immunodeficiencies, and has recently been shown to increase CFTR maturation and to
reduce inflammation in preclinical models of disease. Its mode of action seems to be the
induction of IDO1 which in turns induces autophagy and favourable influencing the balance
of protein folding versus degradation of the CFTR.

There is considerable interest in immunotherapeutic approaches to the treatment of fungal
disease. Recombinant cytokines have been used extensively in transplant-related fungal
disease, and recently successful use of interferon-gamma in CF-related fungal disease was
reported. Targeting the increased inflammasome activation and IL-1β release with the IL-
1-receptor antagonist Anakinra, has shown promising results in pre-clinical models of
disease. Improved understanding of Aspergillus disease pathogenesis in CF patients will
lead to new therapeutic targets and should eventually result in new management options.
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transmembrane conductance regulator determine the functional responses of alveolar

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Table 1. Overview of studies reporting *Aspergillus* colonisation rates in patients with cystic fibrosis.

<table>
<thead>
<tr>
<th>References</th>
<th>Focus</th>
<th>Country</th>
<th>Patient population</th>
<th>Duration and frequency of sampling*</th>
<th>Colonisation rates</th>
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</thead>
<tbody>
<tr>
<td>Nelson, 1979 [22]</td>
<td>primary</td>
<td>USA</td>
<td>46 pts</td>
<td>once</td>
<td>56.7% Asp colonisation (no info if ABPA or sens)</td>
</tr>
<tr>
<td>Laufer, 1984 [23]</td>
<td>primary</td>
<td>USA</td>
<td>100 pts aged 2 – 34 yrs</td>
<td>once</td>
<td>9% Af colonisation overall 10% in those with ABPA</td>
</tr>
<tr>
<td>Bauernfeind, 1987 [24]</td>
<td>secondary</td>
<td>Germany</td>
<td>102 pts</td>
<td>22 mo period, multiple samples</td>
<td>5.9% Af colonisation (no info if ABPA or sens)</td>
</tr>
<tr>
<td>Mroueh, 1994 [25]</td>
<td>primary</td>
<td>USA</td>
<td>236 pts aged 1 – 41 yrs (mean age 14.5 yrs)</td>
<td>retrospective</td>
<td>25.4% overall Af colonisation 19% isolated Af colonisation 6.5% Af colonisation + ABPA based on single cultures</td>
</tr>
<tr>
<td>Flume, 1994 [26]</td>
<td>primary</td>
<td>USA</td>
<td>27 pts prior to lung transplantation</td>
<td>once</td>
<td>63% Asp colonisation (no info if ABPA or sens)</td>
</tr>
<tr>
<td>Cimon, J Med Mycol 1995 [in 27]</td>
<td>primary</td>
<td>France</td>
<td>210 pts</td>
<td>once</td>
<td>21.4% Af pos culture (only 1% ABPA in the study population)</td>
</tr>
<tr>
<td>Becker, 1996 [28]</td>
<td>primary</td>
<td>USA</td>
<td>49 adult pts</td>
<td>1 yr cross-sectional</td>
<td>16% Af colonisation (no data if ABPA or sens) based on single cultures</td>
</tr>
<tr>
<td>Milla, 1996 [29]</td>
<td>primary</td>
<td>USA</td>
<td>212 paediatric pts &gt; 5 yrs mean age 17.2 +/- 9.2 yrs</td>
<td>multiple 1 yr study period</td>
<td>21.2% at least one Asp pos culture</td>
</tr>
<tr>
<td>Burns, 1998 [30]</td>
<td>secondary</td>
<td>USA</td>
<td>465 pts &gt; 6 yrs</td>
<td>once</td>
<td>23.2% Asp colonisation (ABPA or sens not exclusion criteria for</td>
</tr>
<tr>
<td>Study</td>
<td>Type</td>
<td>Country</td>
<td>Participants</td>
<td>Methodology</td>
<td>Results</td>
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<tr>
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<tr>
<td>Burns, 1999 [31]</td>
<td>Secondary</td>
<td>USA</td>
<td>520 pts ≥ 6 yrs of age, FEV1 25%-75% predicted</td>
<td>Twice (baseline and end of study)</td>
<td>25% Asp colonisation baseline 16.2% wk 20 (end of study) (18% vs 8%, tobra vs placebo)</td>
</tr>
<tr>
<td>Bargon, 1999 [32]</td>
<td>Primary</td>
<td>Germany</td>
<td>104 adult pts</td>
<td>Repeated cultures</td>
<td>41.3% Asp colonisation (no info if ABPA or sens)</td>
</tr>
<tr>
<td>Cimon, 2000 [33]</td>
<td>Secondary</td>
<td>France</td>
<td>128 pts (98 adults and 30 paediatric pts)</td>
<td>Longitudinal, 5 yrs, minimum 4 samples/pt</td>
<td>At least one Af pos culture (46.1% at least one Af pos culture (only 4% ABPA in the population studied))</td>
</tr>
<tr>
<td>Hodson, 2002 [34]</td>
<td>Secondary</td>
<td>UK/Ireland</td>
<td>42 (tobramycin) + 37 (colistin) pts ≥ 6 yrs</td>
<td>Once at end of study</td>
<td>5.7% Af pos in tobramycin group 3.2% Af pos in colistin group</td>
</tr>
<tr>
<td>Bakare, 2003 [35]</td>
<td>Primary</td>
<td>Germany</td>
<td>94 pts, median age 28 yrs</td>
<td>Multiple, 6 mo study period</td>
<td>45.7% Af colonisation 24.5% had ≥ 2 Af positive cultures (no info if ABPA or sens)</td>
</tr>
<tr>
<td>Skov, 2005 [36]</td>
<td>Primary</td>
<td>Australia</td>
<td>270 paediatric and adult pts</td>
<td>2 samples/yr 4 yr study period</td>
<td>Increase from 7.4% to 18.8% (based on single Af pos cultures) ABPA 0.3 to 4%</td>
</tr>
<tr>
<td>Chotirmall, 2008 [37]</td>
<td>Primary</td>
<td>Irish</td>
<td>50 pts</td>
<td>During exacerbations 5 yrs study period</td>
<td>30% at least one Asp positive culture 20% &gt; 1 Asp pos culture 4% ≥ 10 Asp pos culture 12% ABPA</td>
</tr>
<tr>
<td>Valenza, 2008 [38]</td>
<td>Primary</td>
<td>Germany</td>
<td>60 adult and paediatric pts [median 18 yrs, 6 -41 yrs]</td>
<td>Multiple samples 1 yr study period</td>
<td>58.3% A. fumigatus pos 10% non-fumigatus Aspergillus pos (based on single positive cultures)</td>
</tr>
<tr>
<td>Paugam, 2010 [39]</td>
<td>Primary</td>
<td>France</td>
<td>201 adult pts</td>
<td>Multiple samples 2 yrs study period</td>
<td>56.7% in total study population (based on one Af pos culture) 28% age group 6-10 yrs</td>
</tr>
<tr>
<td>Study</td>
<td>Type</td>
<td>Country</td>
<td>Age Group</td>
<td>Follow-up</td>
<td>Findings</td>
</tr>
<tr>
<td>------------------------</td>
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</tbody>
</table>
| Nagano, 2010 [40]     | Secondary| Ireland | 11-15 yrs | once      | 59% age group 11-15 yrs  
75% age group 16-20 yrs  
61% age group 21-41 yrs |
| Jubin, 2010 [41]      | Primary  | France  | 16-20 yrs | multiple  | 77 adult pts, median 28.5 yrs [18-59 yrs]  
once 9.1% all Asp species  
5.2% A. fumigatus (no info ABPA or sens) |
| Sudfeld, 2010 [42]    | Primary  | US      | 10-14 yrs | 10 yr     | 214 pts, median age 28.5 yrs [18-59 yrs]  
multiple at least once/yr  
6 yrs study period  
21% Asp colonisation  
14% isolated persistent Asp colonisation  
4% Asp colonisation + ABPA |
| De Vrankrijker, 2011 [43] | Primary  | Netherlands | 16-20 yrs | 10 yr     | 259 pts non-ABPA  
106 pts 0-12 yrs  
99 pts 13-24 yrs  
54 pts ≥25 yrs  
multiple in one yr  
23.6% (>50% of cultures Af pos)  
16.4% age group 0-12 yrs  
54.1% age group 13-24 yrs  
29.5% age group ≥ 25 yrs |
| Wainwright, 2011 [44] | Secondary| Australia| 5-16 yrs  | during pulmonary exacerbations | 93 pts, BAL fluid from children < 5 yrs  
9% Asp colonisation/infection |
| Fillaux, 2012 [45]    | Primary  | France  | 16-20 yrs | 10 yr     | 206 pts median age 16.3 yrs [range 9.8-23.6]  
routine samples, median follow-up 3.6 yrs [range 2.1-8.7]  
18% persistent carriage = 3 Af pos cultures in 6 mo  
62.1% at least one Af pos culture  
27.1% persistent carriage period at least once |
| Güngör, 2013 [46]     | Primary  | Turkey  | 11-14 yrs | at least 3 sputum/deep throat swab samples | 48 pts, mean age 11.6 yrs [range 2-38 yrs]  
10.4% one A. fumigatus pos culture  
8.3% non-fumigatus Aspergillus (no info ABPA or sens) |
<table>
<thead>
<tr>
<th>Study</th>
<th>Setting</th>
<th>Country</th>
<th>Age/Number</th>
<th>Methodology</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fillaux 2014 [47]</td>
<td>primary</td>
<td>France</td>
<td>117 pts median age 9.9 yrs [IQR 6.0-13.2]</td>
<td>routine 8 yrs period</td>
<td>38.5% (= 3 Af pos cultures in 6 mo) without sensitization</td>
</tr>
<tr>
<td>Masoud-Landgraf, 2014 [48]</td>
<td>secondary</td>
<td>Austria</td>
<td>113 pts, median 20.3 yrs [range 2-57]</td>
<td>multiple samples 1 yr study period</td>
<td>78.8% Af pos cultures (no info ABPA or sens)</td>
</tr>
<tr>
<td>Noni, 2015 [49]</td>
<td>primary</td>
<td>Greece</td>
<td>121 paediatric pts</td>
<td>routine</td>
<td>32.2% one Af pos culture 11.6% chronic colonisation</td>
</tr>
<tr>
<td>Ramsey, 2014 [6]</td>
<td>primary</td>
<td>Australia</td>
<td>56 infants ≤ 2 yrs of age</td>
<td>3 BAL samples 2 yrs study period</td>
<td>12.5% Asp colonisation</td>
</tr>
<tr>
<td>Heltshe, 2015 [50]</td>
<td>secondary</td>
<td>USA</td>
<td>151 pts: 38 pts 6-11 yrs 32 pts 12-17 yrs 81 pts ≥18 yrs</td>
<td>3 cultures as per study protocol</td>
<td>~15% and ~7.5% Asp pos cultures before and after ivacaftor (no info ABPA or sens)</td>
</tr>
<tr>
<td>Saunders, 2016 [51]</td>
<td>primary</td>
<td>UK</td>
<td>45 children &lt; 18 yrs of age</td>
<td>multiple</td>
<td>29% Af pos BAL culture 14% Af pos sputum 42% one Af pos culture 22% persistent colonisation</td>
</tr>
<tr>
<td>Mirkovic, 2016 [52]</td>
<td>primary</td>
<td>Ireland</td>
<td>48 pts</td>
<td>8 sputum samples 2 year period</td>
<td>60.9% in Af sens pts 24% in non-sens pts (at least one pos)</td>
</tr>
<tr>
<td>Gernez, 2016 [53]</td>
<td>primary</td>
<td>US &amp; Ireland</td>
<td>48 and 26 adult pts</td>
<td>At least 2 pos sputum cultures within previous 2 yrs</td>
<td>US 27.1% Af pos Ireland 30.8% Af pos</td>
</tr>
<tr>
<td>Reece, 2017 [54]</td>
<td>primary</td>
<td>Ireland</td>
<td>CF registry 749 pts median 18.1 yrs [range 4-</td>
<td>retrospective, 1 yr study period</td>
<td>11% overall Af colonisation (5% persistent and 6% intermittent) ABPA 5.9%</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Location</th>
<th>Registry</th>
<th>Population</th>
<th>Study Period</th>
<th>Positive Culture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brandt, 2018 [55]</td>
<td>2018</td>
<td>Germany</td>
<td>CF Registry</td>
<td>2599 pts (mean age 21 yrs +/- 12 yrs)</td>
<td>1 yr study period</td>
<td>≤ 10 yrs of age: 0 – 10%&lt;br&gt;11-20 yrs of age: 10-30%&lt;br&gt;21-30 yrs: 7-18%&lt;br&gt;≥31 yrs: 0-18%&lt;br&gt;32.5% at least one Af pos culture/yr (no info if ABPA or sens)</td>
</tr>
<tr>
<td>Hong, 2018 [56]</td>
<td>2018</td>
<td>US</td>
<td>CF Foundation registry</td>
<td>16,095 pts (6 - 45 yrs)</td>
<td>Retrospect 6 yrs study period at least 2 cultures/yr, Asp prevalent cases excluded</td>
<td>27.9% Asp pos overall&lt;br&gt;9.6% persistent Asp pos (≥2 pos cultures during 12 mo; of which 5.4% ABPA)&lt;br&gt;18.4% transient of which 3.4% ABPA</td>
</tr>
<tr>
<td>Coron, 2018 [57]</td>
<td>2018</td>
<td>France</td>
<td>243 pts &gt; 6 yrs (mean age 21.2 +/- 8.7 yrs)</td>
<td>prospective, samples annually or during exacerbation 3 yr study period</td>
<td>35.4% one Af pos culture adults 35.6% children 34.8%</td>
<td></td>
</tr>
</tbody>
</table>

*Based on sputum samples unless indicated otherwise; #patients intermittently or persistently colonised with *A. fumigatus* showed no increased prevalence of ABPA; Af, *Aspergillus fumigatus*; Asp, *Aspergillus* species; ABPA, allergic bronchopulmonary aspergillosis; sens, fungal sensitisation; pos, positive;