# Coinfection with *Pseudomonas aeruginosa* and *Aspergillus fumigatus* in cystic fibrosis

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**A complex relationship exists between *P. aeruginosa* and *A. fumigatus* and is associated with worsened clinical disease in cystic fibrosis. Further study of organism interactions, longitudinal outcomes and therapeutic strategies is warranted.** https://bit.ly/2A161bh

**Cite this article as:** Keown K, Reid A, Moore JE, *et al*. Coinfection with *Pseudomonas aeruginosa* and *Aspergillus fumigatus* in cystic fibrosis. *Eur Respir Rev* 2020; 29: 200011 [https://doi.org/10.1183/16000617.0011-2020].

ABSTRACT

**Objectives:** Cystic fibrosis (CF) lung disease is characterised by mucus stasis, chronic infection and inflammation, causing progressive structural lung disease and eventual respiratory failure. CF airways are inhabited by an ecologically diverse polymicrobial environment with vast potential for interspecies interactions, which may be a contributing factor to disease progression. *Pseudomonas aeruginosa* and *Aspergillus fumigatus* are the most common bacterial and fungal species present in CF airways respectively and coinfection results in a worse disease phenotype.

**Methods:** In this review we examine existing expert knowledge of chronic co-infection with *P. aeruginosa* and *A. fumigatus* in CF patients. We summarise the mechanisms of interaction and evaluate the clinical and inflammatory impacts of this co-infection.

**Results:** *P. aeruginosa* inhibits *A. fumigatus* through multiple mechanisms: phenazine secretion, iron competition, quorum sensing and through diffusible small molecules. *A. fumigatus* reciprocates inhibition through gliotoxin release and phenotypic adaptations enabling evasion of *P. aeruginosa* inhibition. Volatile organic compounds secreted by *P. aeruginosa* stimulate *A. fumigatus* growth, while *A. fumigatus* stimulates *P. aeruginosa* production of cytotoxic elastase.

**Conclusion:** A complex bi-directional relationship exists between *P. aeruginosa* and *A. fumigatus*, exhibiting both mutually antagonistic and cooperative facets. Cross-sectional data indicate a worsened disease state in coinfected patients; however, robust longitudinal studies are required to derive causality and to determine whether interspecies interaction contributes to disease progression.

## Introduction

Cystic fibrosis (CF) is the most common inherited lung disease worldwide, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Resultant dysfunctional, or absent CFTR protein on the apical airway epithelial membrane, leads to anion depletion and reduction in the

Submitted article, peer reviewed

Received: 14 Jan 2020 | Accepted after revision: 16 May 2020

airway surface liquid, causing a triad of mucus stasis, chronic infection and inflammation in the CF airways. Over the past decade there has been much focus on the development of small molecule therapies that are capable of restoring CFTR function. While these therapies may be transformational for some people with CF, individual responses may be variable and improved CFTR function does not halt inflammation, eradicate microbial pathogens residing in the airways or reverse existing lung damage [1–4].

In addition to the traditional CF respiratory pathogens such as *Pseudomonas aeruginosa*, *Staphlococcus aureus*, *Haemophilus influenzae* and *Burkholderia cepacia complex*, recent metagenomic microbiome studies have identified a much more diverse airway microbial environment than was previously thought [5]. Rich communities of aerobic and anaerobic bacteria, viruses and fungi coinhabit the airways, with a dynamic composition and waning diversity as CF lung disease progresses [6, 7]. These new insights have led to the identification of novel pathogens such as *Ralstonia mannitolilytica, Prevotella* spp. and *Veillonella* spp. [5, 8, 9] and raised questions of how previously overlooked organisms and interspecies interactions may influence disease progression.

The Gram-negative bacterium *P. aeruginosa* contributes significantly to respiratory morbidity and mortality in CF lung disease [10]. The presence of several organisms coinhabiting the CF airways have been shown to influence the virulence of *P. aeruginosa*, producing both gainful or inhibitory effects [11–17]. However, the potential for interspecies interactions is vast and we are at an early stage in our understanding of this novel aspect of pathogenesis in CF lung disease. Despite the demonstration of multiple direct and indirect organism interactions *in vitro*, it remains unclear whether these interactions are clinically significant and how they contribute to disease progression. Here we review the interaction of the most common bacterial and fungal species in the CF respiratory tract, *P. aeruginosa* and *A. fumigatus* [18–20], consider the clinical implications and future directions for management of polymicrobial infections in CF.

## Epidemiology

Because of the physiological basis of the disease, acquisition of microbes occurs from the immediate environment *via* the upper gastrointestinal tract or the upper respiratory tract, through ingestion or inhalation, respectively. Aspiration of microbes may also occur from the gastrointestinal tract to the upper respiratory tract. Combinations of these routes of entry manifest in the presence of a diverse variety of bacteria and fungi in the sputum of CF patients. A comprehensive review on the microbiology of CF has been reported previously [21, 22].

*P. aeruginosa* and *A. fumigatus* represent the most dominant bacterial and fungal species, respectively, within the CF respiratory tract. Presently, there are 255 species described within the genus *Pseudomonas*, of which. *P. aeruginosa* is the most prevalent in patients with CF. Despite the aggressive eradication protocols widely used in CF care, 60–70% of CF patients are intermittent or chronically colonised with *P. aeruginosa* by the age of 20 years [23, 24].

There are approximately 180 species of *Aspergillus* spp., of which *A. fumigatus* is the most common and clinically significant in patients with CF. The prevalence of *A. fumigatus* colonisation in CF patients is between 16% and 58% [25–28], with rising rates of isolation over the past decade [29]. A range of factors including *P. aeruginosa* eradication treatment, frequent courses of antibiotics, prolonged use of inhaled antibiotics, inhaled corticosteroids and the widespread use of azithromycin are related to the early acquisition and rising prevalence of *A. fumigatus* [29–32]. However, variation in diagnostic techniques and surveillance practices between centres is undoubtedly a contributory factor to both the increased and variable prevalence [33]. The use of nonculture-based diagnostic techniques, such as nucleic acid amplification technologies and matrix-assisted laser desorption/ionisation, have been shown to significantly improve detection of fungal organisms over conventional culture techniques [34, 35]; however, access to these technologies may be limited to specialist mycological laboratories [35]

Epidemiological studies indicate there is a wide variation in the prevalence of chronic co-infection with *P. aeruginosa* and *A. fumigatus* of 16–35% reported in the Irish CF population [36] and a recent meta-analysis showed a pooled prevalence of 15.8% with significant variation, ranging between 2.3% and 44.8% among CF patients [37]. Accurate estimation of the prevalence of *P. aeruginosa* and *A. fumigatus* co-infection poses several challenges due to inconsistent definitions relating to fungal disease, nonstandardised diagnostic techniques, sampling frequency and clinical interpretation of culture results between centres.

## Clinical significance of *P. aeruginosa* and *A. fumigatus* in CF

It is well established that *P. aeruginosa* plays a central role in the progression of CF lung disease [24] and it is considered to be the primary pathogen leading to deterioration of lung function, hospitalisation and death in CF [24]. However, the role of *A. fumigatus* is less clearly defined. The ubiquitous environmental

filamentous fungi pose a serious threat causing invasive infection in immunocompromised patients; however, it is effectively cleared in the immunocompetent host through robust multifaceted immune responses. In CF and other chronic respiratory conditions, impaired mucociliary clearance and structural lung damage favours *A. fumigatus* persistence in the airways, furthermore, ineffective clearance of bacterial and fungal pathogens by phagocytes expressing defective CFTR places people with CF at particular risk of *A. fumigatus* infection [38–41].

The findings of several studies have indicated that persistent isolation of *A. fumigatus* is uncommon in children aged <10 years [42–44], as such it has been considered to be a late occurring infection, likely to be acquired as a result of changes in the microbial milieu or disease state over time. However, recent findings from the AREST-CF study group, using early bronchoalveolar lavage (BAL) sample, indicate that *A. fumigatus* is one of the most prevalent organisms isolated from the lower airways of young children [45, 46]. They have also shown that early *Aspergillus* infections are an independent risk factor for the progression of lung disease in children with CF [47]. These findings indicate that the presence of *A. fumigatus* is not dependant on advanced disease states, age of the patient or on the presence of other pathogens as was previously thought.

Several observational studies have demonstrated a worsened clinical condition associated with co-colonisation with *P. aeruginosa* and *A. fumigatus* [25, 36, 48–50]. These include two large cross-sectional registry data studies, which included both paediatric and adult patients. The Irish registry data showed that co-colonisation was associated with reduced forced expiratory volume in 1 s ($FEV_1$), more frequent hospitalisation, more frequent pulmonary exacerbations and increased antimicrobial usage, when compared to those not co-colonised [36], while the UK CF registry showed co-colonised patients had an increased use of intravenous antibiotics without correlating with a lower $FEV_1$ [49]. A further single-centre UK study of adult patients showed they had lower $FEV_1$, more *i.v.* antibiotics and a lower body mass index [50].

## *A. fumigatus*-related lung disease in CF

The hypersensitivity lung disease allergic bronchopulmonary aspergillosis (ABPA) affects up to 10% of people with CF [51]. Diagnostic criteria for ABPA were established in 2003 [27], and are based on a range of clinical, serologic and radiological parameters that continue to be in widespread use today. While there is some variation in management practices, there is consensus amongst clinicians that a diagnosis of ABPA has significant clinical implications that warrants treatment with systemic corticosteroids, with or without antifungal therapy [51, 52]. In addition to ABPA, *Aspergillus*-related lung disease may have other manifestations in people with CF. A classification system proposed by Baxter *et al.* [53] based on a cluster analysis in adults identified four distinct classes of *Aspergillus*-related lung disease in CF: ABPA, *Aspergillus* sensitisation, *Aspergillus* colonisation and *Aspergillus* bronchitis [53].

*Aspergillus* bronchitis refers to fungal infection which is confined to the bronchial tree, causing superficial mucosal invasion and symptoms of cough and increased mucus production and is distinguished from the other entities by qPCR and *Aspergillus* immunoglobulin-G alongside negative serological markers of allergic disease [53]. Its first description was based on a small group of CF patients who were nonresponsive to antimicrobial therapy, who chronically isolated *A. fumigatus* without ABPA and all of whom had a clinical response to antifungal treatment [48]. A proportion of patients exposed to *A. fumigatus* will become immunologically sensitised; these patients more commonly develop ABPA and the immunological response to the fungi may represent a spectrum of hypersensitivity disease. Several studies have shown that *Aspergillus* sensitisation without ABPA has been associated with poorer lung function in its own right [54–56].

While the above classification provides a useful clinical and research framework for *A. fumigatus* lung disease, the proposed biomarkers to differentiate between these clusters have yet to be prospectively validated. Further study of these subgroups, particularly *A. fumigatus*-colonised and bronchitis patients would be beneficial to understand which patients have inconsequential colonisation and which have active fungal infection. Furthermore, while the proposed classification separates hypersensitivity from nonhypersensitivity *Aspergillus* disease, it does not incorporate other manifestations including pulmonary aspergilloma [57] or invasive aspergillosis, which may rarely complicate CF lung disease [58].

Uncertainty around the significance of the chronic isolation of *A. fumigatus* is fuelled by conflicting evidence around its direct effect on progression of lung disease. Several studies show that it does not directly affect absolute $FEV_1$ [42, 43, 59]; however, a number of other studies indicate that it is independently associated with a lower and more accelerated decline in $FEV_1$ [25, 29, 60–62]. Additionally, it has been shown to be associated with more frequent pulmonary exacerbations [25], more advanced bronchiectasis on high-resolution computed tomography (HRCT) [63], elevated BAL neutrophil count [64] and persistent inflammation in a CF murine model [65, 66].

## Survival of *P. aeruginosa* and *A. fumigatus* in CF airways

Over the course of chronic infections, *P. aeruginosa* displays a range of mechanisms, phenotypic and genetic adaptations which enable it to persist in the CF lung. Early in infection, *P. aeruginosa* releases virulence factors which enable it to overcome host defences and establish infection, they include toxic phenazines and rhamnolipids, which promote ciliary stasis [67], and proteases, including LepA, which activate nuclear factor (NF)-kB to increase inflammation [68]. Over time, *P. aeruginosa* maintains infection through release of immunosuppressing factors (exoproteins) such as the elastases LasA and LasB, which cleave the connective tissue protein elastin and the immune modulator surfactant protein D [69]. It also mutates into small-colony variants (SCVs), mucoid strains and forms impenetrable biofilms, which create a physical and chemical barrier against antimicrobial agents and the host immune system [70].

On exposure to *A. fumigatus*, alveolar macrophages recognise fungal surface antigens (galactomannan, β-d-glucan) through alveolar macrophage surface receptors such as Dectin-1 and Toll-like receptors. Recognition of *A. fumigatus* leads to production of proinflammatory cytokines through activation of the NF-kB and inflammasome pathways, triggering an influx of neutrophils, natural killer and T-cells to the site of infection. T-helper (Th) cells are differentiated into a predominant Th1 response with generation of tumour necrosis factor-α and interferon-γ .

*A. fumigatus* has a range of immune-evasion strategies to avoid clearance by the host protective responses, primarily through the release of secondary metabolites, including mycotoxins and, like *P. aeruginosa* it forms biofilms. Gliotoxin is the most abundant mycotoxin released by *A. fumigatus*, its action is through suppressing immune responses including NF-kB [71], macrophage phagocytosis [72], T-cell function [73] and neutrophil activation [74]. Furthermore, gliotoxin may impair the integrity of the epithelial cell wall and has been shown to kill lung epithelial cells *in vitro* [75].

## Interactions between *P. aeruginosa* and *A. fumigatus*

### *Inhibition by* P. aeruginosa

In CF, *P. aeruginosa* inhibits *A. fumigatus* through a range of different mechanisms and to a greater extent than in non-CF isolates [76]. The primary inhibitory mechanism is through the release of the virulence factors, phenazines. These include pyocyanin (PYO), phenazine-1-carboxamide, 1-hydroxyphenazine (1-HP) and phenazine-1-carboxylic acid (PCA), which promote *P. aeruginosa* growth and are toxic to surrounding bacteria, fungi and mammalian cells [77]. Phenazines inhibit the growth of *A. fumigatus* through the generation of reactive oxygen species (ROS) and reactive nitrogen species, which damage the mitochondrial ultrastructure of *A. fumigatus* hyphae [78]. In addition to causing oxidative stress to the lung, PYO is directly toxic to cilia, upregulates interleukin (IL)-8 activity, causes cellular senescence [78–82] and inactivates $\alpha_1$-antitrypsin, an important component of the endogenous antiprotease shield, contributing to protease/antiprotease imbalance within the lung. PYO is regulated by the *P. aeruginosa* quorum sensing (QS) system [77] and levels have been directly correlated with prognosis [83] and frequency of pulmonary exacerbations [84].

The QS system allows bacteria to sense each other and to regulate physiological activities such as virulence, motility and biofilm formation through small diffusible signalling molecules, which modulate the pathogenicity of microorganisms found in the CF respiratory tract. The role of the QS in *A. fumigatus* inhibition was demonstrated recently by Sass *et al.* [85] in *P. aeruginosa* QS knockout strains, showing that *A. fumigatus* growth was significantly higher than when in direct co-culture with wild-type *P. aeruginosa*, confirming inhibition of *A. fumigatus* by *P. aeruginosa via* QS. Furthermore, the viability of conidia and *A. fumigatus* biofilm mass was reduced by diffusible and heat-soluble molecules released by *P. aeruginosa*, which are structurally similar to QS molecules[86], though the effect was less pronounced in established mixed-species biofilms [80, 85, 86].

The QS system also controls the production of the *P. aeruginosa* virulence molecules, rhamnolipids. These induce *A. fumigatus* production of an extracellular matrix that inhibits *A. fumigatus* growth by altering cell wall architecture [87]. *P. aeruginosa* also secrete the interkingdom signalling molecules alkylhydroxyquinolones [88], produced in response to increasing density of bacterial cells, which influence gene expression, phenazine secretion [80] and have been shown to disrupt *A. fumigatus* biofilm integrity [88].

In addition to intermicrobial signals and the release of redox-active toxins, nutrient competition is a further mechanism of *P. aeruginosa* inhibition of *A. fumigatus* growth. Iron is a central micronutrient for the survival of both *P. aeruginosa* and *A. fumigatus*, with a particular role in biofilm formation [89]. *P. aeruginosa* produces the siderophore, pyoverdine [90], which captures iron from the environment and stores it. Through iron deprivation, pyoverdine has a substantial antifungal activity [78, 85, 86]. Sass *et al.* [90] have shown that *P. aeruginosa* mutants lacking pyoverdine have less inhibitory capacity for *A. fumigatus* growth, indicating the importance of pyoverdine as a means of *A. fumigatus* inhibition.

Similarly, the production of siderophores has also been shown to be increased in the presence of other competing bacterial organisms, including *S. aureus* [91] and *Burkholderia* spp. [92].

Denial of iron to *A. fumigatus* is also the mechanism of inhibition by the *P. aeruginosa*-produced bacteriophage Pf4 [93]. This endogenous phage inhibits the metabolic activity of *A. fumigatus* biofilms and was more pronounced against preformed *A. fumigatus* biofilm rather than biofilm formation, while conidial growth was unaffected. The authors also demonstrated that the inhibition of *A. fumigatus* metabolism by Pf4 could be overcome with supplemental ferric iron, again demonstrating the central role this micronutrient in bacterial–fungal competition.

### *Reciprocal antagonism by* A. fumigatus

Despite a range of antagonistic mechanisms and fungicidal properties of *P. aeruginosa*, *A. fumigatus* manages to survive in CF airways in close proximity to *P. aeruginosa* within the shared ecosystem of the CF airways. Investigation of the antifungal properties of *P. aeruginosa* has shown that *P. aeruginosa* clinical isolates fail to completely inhibit *A. fumigatus* [85, 94]. These findings illustrate that *A. fumigatus* has the ability to counteract antagonistic actions of *P. aeruginosa* and that the relationship may shift between antagonism and cooperation.

Variation in the inhibitory capacity of *P. aeruginosa* has been demonstrated though several studies. Mowat *et al.* [86] showed that once filamentous *A. fumigatus* biofilms have been produced, the inhibitory capacity of *P. aeruginosa* is significantly restricted through small diffusible and heat stable molecules. The antifungal capacity of *P. aeruginosa* also diminishes as *A. fumigatus* condita transition into hyphae as their walls become impermeable to *P. aeruginosa* metabolites and the antibacterial mycotoxin, gliotoxin, is released [72, 95, 96]. This was demonstrated in the *Galleria mellonella* infection model, indicating that *P. aeruginosa* and *A. fumigatus* exert mutual antagonism within shared biofilms [96].

As a further line of defence against the antifungal effects of *P. aeruginosa*, *A. fumigatus* produces its own siderophores, allowing it to preserve iron, a vital capability for survival in iron-scarce conditions, such as during pulmonary exacerbations or advanced lung disease. The central role of these siderophores was confirmed using *A. fumigatus* mutant strains lacking the *SidA* gene, which exhibited less capacity to preserve *A. fumigatus* biofilms than *A. fumigatus* wild type when exposed to the toxic phenazine pyoverdine [90].

Toxic phenazines produced by *P. aeruginosa*, inhibit *A. fumigatus* in high concentrations through production of reactive oxygen and nitrogen species; however, concentrations of PYO and PCA occur in the range of 1–100 µM in CF sputum samples, these concentrations have been demonstrated to be to be subinhibitory to *A. fumigatus* [78]. Furthermore, in the presence of low concentrations of phenazines in CF airways, iron bioavailability is enhanced, thereby sustaining *A. fumigatus* biofilms. *A. fumigatus* has also been shown to have the ability to bio-transform phenazines into alternative forms with more favourable properties, including PCA conversion to 1-HP, which induces *A. fumigatus* siderophore production [80]. These findings demonstrate the complex interplay between these organisms and how *A. fumigatus* has mechanisms to evade antagonism by *P. aeruginosa*.

### *Cooperation*

The antagonistic and counter-antagonistic mechanisms enable both organisms to coexist; however, beyond tolerance of each other, cooperative, virulence-enhancing effects have also been demonstrated. As *A. fumigatus* infection is found in many CF patients following *P. aeruginosa* infection [97], it is likely that *P. aeruginosa* facilitates the establishment and growth of *A. fumigatus*. As described, one mechanism facilitating this is the sub-bacteriostatic airway concentrations of phenazines, which induce *A. fumigatus* growth through increasing iron bioavailability [78].

Volatile organic compounds released by *P. aeruginosa* can communicate at a distance with *A. fumigatus*, without direct contact with the effect of promoting fungal growth [98]. The compound dimethyl sulphide mediates this effect through communication in the gas phase, thus *P. aeruginosa* may create an environment which is conducive to inhabitation by *A. fumigatus* and precipitate fungal growth once infection is established. It has been shown that the phenazine 1-HP is able to chelate iron [78] thereby contributing to iron starvation in *A. fumigatus*. However, it has also been shown that the iron chelating activity of 1-HP induces the transcription of genes for adaptation to iron starvation in *A. fumigatus*, demonstrating the adaptive capability of *A. fumigatus* in the presence of *P. aeruginosa* [78, 99].

*P. aeruginosa* also gains from the presence of *A. fumigatus*. In the *Galleria mellonella* insect model, Reece *et al.* [96] showed that *P. aeruginosa* had enhanced killing capacity when pre-exposed to *A. fumigatus* larvae. Within *in vitro*-mixed *P. aeruginosa and A. fumigatus* biofilms, *P. aeruginosa* displayed increased antimicrobial resistance, when compared to *P. aeruginosa* in monomicrobial biofilms, likely due to altered permeability of the biofilm extracellular matrix, however the same was not observed of *A. fumigatus*

antifungal susceptibility, which showed no difference between mixed and monomicrobial biofilms [100]. This work indicates that co-presence of *A. fumigatus* may accelerate phenotypic adaptations and genetic mutations in *P. aeruginosa*, thereby enhancing virulence of the organism.

## Relationship dynamics over time and disease course

As we have described, the relationship between *P. aeruginosa* and *A. fumigatus* is complex with potential for both inhibitory and cooperative interactions (figure 1). Mutual antagonism allows each organism to coexist despite the hostile conditions of the CF airways, theoretically maintaining balance by preventing proliferation of either organism. However, in a cooperative state, enhanced virulence of these organisms may contribute to disease progression.

The factors influencing shifts between antagonism and cooperation in the co-infection state are not clear. Severity of CF lung disease may be one of the factors determining the nature of species interaction. In hypoxic, anaerobic conditions, the inhibitory effect of phenazines on both planktonic and biofilm forms of *A. fumigatus* is diminished [101]. Regional ventilation inhomogeneity exists in the CF lung where mucus impaction and bronchiectasis occur. These findings indicate that advancing lung disease or pulmonary exacerbation may favour *A. fumigatus* growth. A further example of the variation of inhibition in different infection stage was demonstrated in *P. aeruginosa* SCVs, which showed variation in inhibitory capacity towards *A. fumigatus* which is directly related to levels of pyoverdine production [102].

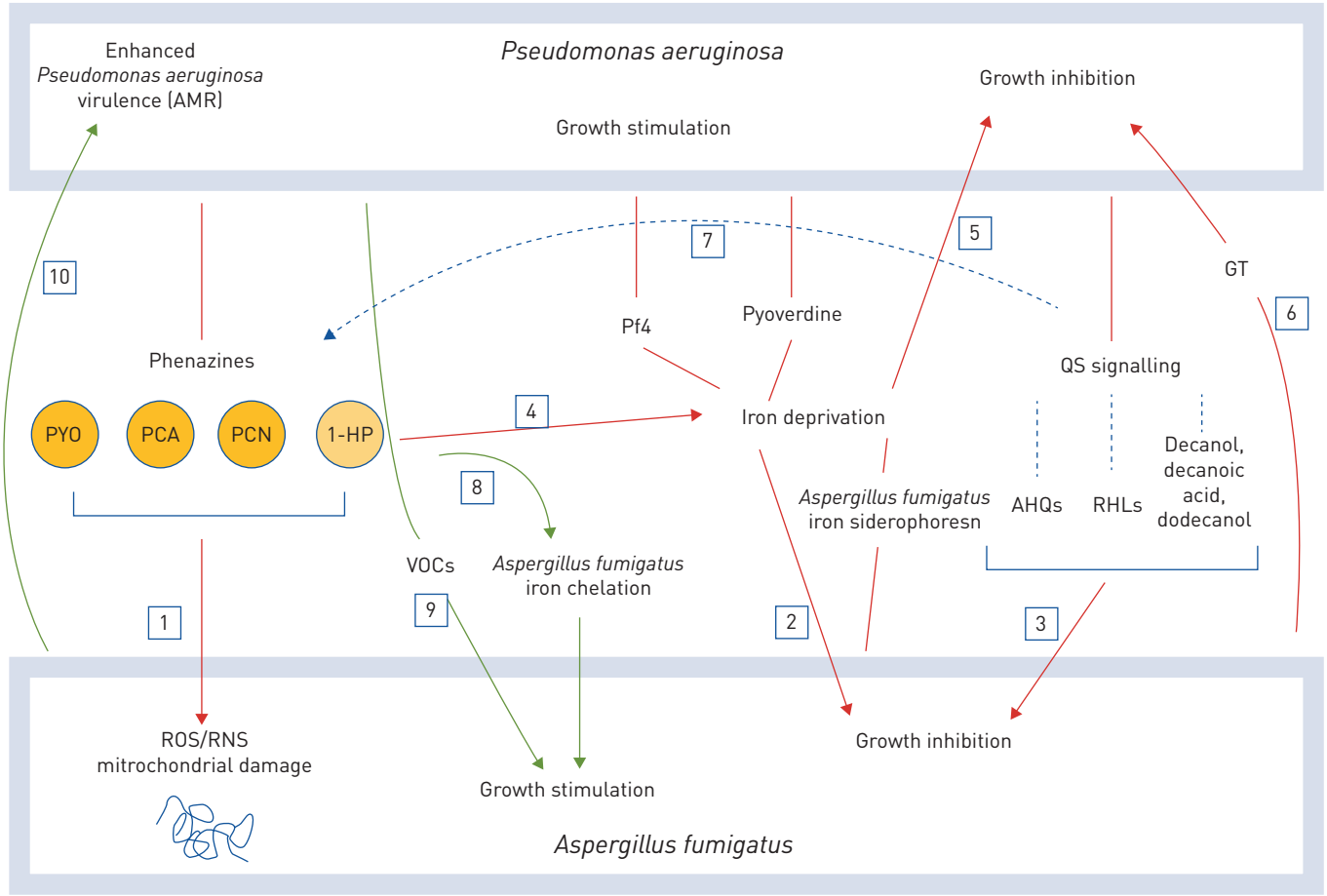


FIGURE 1 Interactions between *Pseudomonas aeruginosa* and *Aspergillus fumigatus*. Inhibition is shown by red arrows. 1) Phenazines inhibit *A. fumigatus* growth through generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) which damage *A. fumigatus* mitochondrial ultrastructure. 2) Pyoverdine and bacteriophage Pf4. 3) *P. aeruginosa* quorum sensing system (QS). 4) Phenazine 1-hydroxyphenazine (1-HP) chelates iron, contributing to *A. fumigatus* iron deprivation. 5) *A. fumigatus* siderophores compete with *P. aeruginosa* siderophores for iron. 6) Gliotoxin (GT), a primary mycotoxin released by *A. fumigatus*. Regulation is shown by black arrow. 7) QS signalling controls phenazine release. Stimulation is shown by green arrows. 8) Subinhibitory phenazine levels promote *A. fumigatus* iron availability. 9) Volatile organic compounds (VOCs) released by *P. aeruginosa* stimulate *A. fumigatus* growth. 10) *P. aeruginosa* phenotypic adaptations influenced by *A. fumigatus*, including antimicrobial resistance (AMR). PYO: pyocyanin; PCA: phenazine-1-carboxylic acid; PCN: phenazine-1-carboxamide; AHQ: alkylhydroxyquinolones; RHL: rhamnolipids.

The nature of the interspecies relationship may also vary between planktonic and biofilm forms of infection. This was demonstrated through the finding that pyoverdine production by *P. aeruginosa* in biofilms is higher than in the planktonic state [76, 103]. However, several studies have demonstrated that while *P. aeruginosa* can inhibit *A. fumigatus* biofilm formation, its inhibition is rather ineffective on established biofilms [76, 86]. In monomicrobial *A. fumigatus* biofilms, metabolic activity of the fungus progressively wanes as germinating conidia transition to hyphae and form mature filamentous biofilms [86]. This lower metabolic state desensitises *A. fumigatus* to *P. aeruginosa* phenazines, whose action is concentrated in metabolically active sites. Other conditions including host immunity, comorbidity and exogenous factors, such as the use of antibiotics or corticosteroids, may influence interspecies interaction; however, these factors have yet to be evaluated.

## Host immunity and inflammation

It is known that co-infection with *P. aeruginosa* and *S. aureus* has an additive effect on endobronchial inflammation. Furthermore, an intensifying degree of inflammation has been observed with rising number of species in polymicrobial infections [104]. However, effects of cross-kingdom polymicrobial infection on airway inflammation have not been examined. Although ineffective clearance of bacterial pathogens by CFTR defective phagocytes and other immune mechanisms have been well demonstrated [39, 40, 105], only a few studies have examined the immune responses of CFTR defective immune cells to *A. fumigatus* and other fungal pathogens [38, 106, 107].

Individually, both *P. aeruginosa* and *A. fumigatus*, are capable of inducing proinflammatory responses in the lung epithelium. Inhaled conidia can be cleared without evoking any immune response; however, in the transition into hyphae, proinflammatory cell surface constituents promote phagocyte activity [108]. This was demonstrated in a CF mouse model where *A. fumigatus* elicited hyperinflammatory responses in airway epithelial cells [107], with elevated percentages of macrophages, neutrophils and neutrophilic chemokines in BAL fluid within 24 h of exposure of $CFTR^{-/-}$ mutants to conidia. Furthermore, in that study, $CFTR^{-/-}$ mice were unable to effectively clear conidia by 6 h, in contrast to the complete clearance by wild-type mice. Further murine models have demonstrated release of IL-1b, exaggerated neutrophil response [66], and profound Th2 hyperinflammatory response to *A. fumigatus* antigens [109]. These findings illustrate the proinflammatory effects and dysregulated immune responses in response to *A. fumigatus*, and the effects of impaired innate antifungal immunity in *Aspergillus* infections in CF.

There is an intense, neutrophilic inflammation present in CF airways with abundant degranulating neutrophils overwhelming endogenous antiprotease mechanisms, thus causing a protease–antiprotease imbalance [110]. It has been shown that *A. fumigatus* enhances the production of *P. aeruginosa* cytotoxic elastase, adding to this imbalance and causing direct epithelial damage, mucociliary and CFTR dysfunction [111, 112]. However, Reece *et al.* [96] did not find elevation of IL-6 and IL-8 levels in co-cultures, compared to infection with *P. aeruginosa* alone, possibly related to the effect of mutual antagonism, suppression of immune responses by organisms or saturated pathways for cytokine production.

It has recently been shown that the fungal recognition receptor Dectin-1 is cleaved by elastase in BAL fluid, leading to under-detection of the arrival of fungal pathogens in the lung [113]. This work indicates for the first time that neutrophil derived proteases lead to *A. fumigatus* persistence and infection through inactivation of fungal receptors; the effects of other proteases has not been examined.

With clear inflammatory implications and likely contribution to protease–antiprotease imbalance, examination of inflammatory effects and a better understanding of the innate immune responses is needed to better understand how these pathogens and their interaction influence disease progression.

## Should we treat *A. fumigatus* in coinfected patients?

Combined antimicrobial and antifungal treatment in patients chronically coinfected with *P. aeruginosa* and *A. fumigatus* has not been evaluated in clinical trials. Baxter *et al.* [114] showed that the use of short-term *i.v.* antibiotics, administered during pulmonary exacerbation to target *P. aeruginosa*, reduced the quantities of both *P. aeruginosa* and *A. fumigatus* isolated in sputum at the end of treatment by PCR. The reason for this is unclear; however, the authors postulate that *P. aeruginosa* biofilms may protect and sustain *A. fumigatus* from host immunity and in provision of nutrients.

The benefits of use of antifungal therapies in *A. fumigatus* disease is unclear due to insufficient existing research. There is a single randomised controlled trial that evaluates the effect of eradication of *A. fumigatus* in 35 chronically infected patients with 24 weeks of itraconazole or placebo. The study found no improvement in frequency of pulmonary exacerbations or $FEV_1$ [115], which supports a conservative clinical management strategy. However, a major flaw of this study was failure to achieve therapeutic antifungal levels in the majority of patients.

Three further studies, all case–series or cohort studies without randomisation, have evaluated the effect of different azoles in CF patients chronically colonised with *A. fumigatus*. The first included a small number of people with CF with chronic *A. fumigatus* in their sputum (without serological evidence of ABPA) treated with voriconazole for a median of 22 weeks. There was no change in $FEV_1$ following antifungal treatment and serum drug levels were not tested in any of the patients [116]. In contrast to the above findings, two small studies using posaconazole [117] and itraconazole [118] showed that eradication of *A. fumigatus* in chronically colonised patients resulted in fewer pulmonary exacerbations, improved respiratory symptoms, lung function and in one study improvements were seen on HRCT [118].

The effect of treatment in *Aspergillus*-sensitised and *Aspergillus* bronchitis patients has received even less attention. Two small studies have evaluated the effect of antifungal therapy in *Aspergillus* bronchitis patients [48, 119]; both showed improved respiratory symptoms and improvement in lung function. In *Aspergillus*-sensitised patients, Kanthan *et al.* [120] retrospectively compared two cohorts of sensitised children; the second cohort, who received more antifungal treatment had higher $FEV_1$ than the second cohort. However, this was a low-quality study comparing groups of patients from different time-points with variable rates of ABPA between the two cohorts . Even within ABPA, the role of antifungal therapy is unclear with variations in clinical practice amongst clinicians [52]. A systematic review of antifungal treatment in ABPA including four randomised controlled trials, showed that symptoms, frequency of pulmonary exacerbations and lung function all improved with antifungal treatment. [121]. However, adverse effects were common, therefore recommendations for use of antifungals in ABPA is classed as "weak" by the British Society for Allergy and Clinical Immunology.

In the absence of clear data on the effectiveness, safety and tolerability of antifungal therapies in chronically infected patients and across the different *Aspergillus* phenotypes, clinicians have to weigh up the potential treatment gains against the significant and well-documented adverse effects of long-term treatment, including toxicity, drug interactions and the emerging issue of azole resistance. Furthermore, to warrant such treatment, clinicians need to be certain about the clinical implications of *A. fumigatus* colonisation. Those coinfected with *A. fumigatus* and *P. aeruginosa* represent a particular subgroup of patients in whom complex mechanistic interactions appear to confer a worse disease state, in whom targeted antifungal treatment is likely to be beneficial.

Going forward, there is a need for robust longitudinal data and identification of biomarkers to separate "harmless" colonisation from those which have active *Aspergillus* infection. This needs to be followed by robust therapeutic antifungal trials to determine the optimal antifungal treatment across different subsets of patients (*i.e.* those with *Aspergillus* colonisation, bronchitis and sensitisation). Novel treatment strategies must also be evaluated, which may obviate the need for prolonged toxic antifungal treatment, including anti-inflammatory treatments and immunotherapeutic approaches. Although evaluation of a range of different anti-inflammatory therapies is ongoing in CF, in-depth characterisation of the inflammatory implications of this co-infection may identify potential pathways for targeted anti-inflammatory treatments. Recent preclinical evaluation of anakinra, the IL-1-receptor antagonist has shown potential in reducing inflammation through inhibition of IL-1b and correction of dysregulated inflammasome responses [66]. Finally, the effects of CFTR modulator treatments on polymicrobial infections warrants close evaluation, as a reduction in fungal colonisation has been observed following ivacaftor treatment in patients with G551D [122], likely due to complete or partial restoration of CFTR function in innate immune cells.

## Conclusion

It is clear that *P. aeruginosa* and *A. fumigatus* interact through a range of mechanisms, producing a variably competitive and cooperative relationship, enabling organisms to coexist and thrive in a shared habitat. However, there are many gaps in our understanding of how this relationship evolves over time and disease state and crucially, the clinical implications of this interaction. There is an urgent need for standardisation of terminology, definitions and culture techniques in relation to fungal infection in CF, to enable robust longitudinal studies to be performed and to explore novel therapeutic strategies. Furthermore, clinically accessible biomarkers are needed to identify those most significantly affected by direct targeted treatment in co-colonised patients.

Conflict of interest: None declared.

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