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Review

What is the clinical significance of filamentous fungi positive sputum cultures in patients with cystic fibrosis? ☆

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Abstract

In patients with cystic fibrosis (CF), the isolation of filamentous fungi, in particular *Aspergillus* spp. in the respiratory secretions is a common occurrence. Most of these patients do not fulfil the clinical criteria for a diagnosis of allergic bronchopulmonary aspergillosis (ABPA). The clinical relevance of filamentous fungi and whether antifungal therapy should be started in patients with persistent respiratory exacerbations who do not respond to two or more courses of appropriate oral or intravenous antibiotics and in whom no other organisms are isolated from respiratory secretions is a dilemma for the CF clinician. In this article, we review the epidemiology and clinical significance of filamentous fungi in the non-ABPA CF lung, with an emphasis on *Aspergillus* spp. colonisation (AC), the clinical relevance of *Aspergillus* spp. positive respiratory cultures and the outcome following antifungal therapy in these patients.

Keywords: Cystic fibrosis; Children; Adults; Sputum; Fungi; Aspergillus spp.; Laboratory

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1. Introduction

Cystic fibrosis (CF) is a genetic disease that affects over 8500 people in the UK and over two million people carry the gene that causes CF. As a result of new therapies and more aggressive antibiotic management, life expectancy for children born in this decade is expected to be 50 years or more [1]. Patients usually die early as a result of progressive lung disease caused by recurrent and ultimately chronic respiratory infections. The condition is a result of a mutation in the cystic fibrosis transmembrane regulator gene on chromosome 7 which encodes a chloride channel. Defective channel activity leads to thick, viscous secretions and impaired mucociliary clearance in the lung [2]. This causes trapping of mucus, colonisation with bacteria and fungi, and a persistent inflammatory response in the airways. Patients are prone to infection with common respiratory pathogens such as *Haemophilus influenzae* or *Staphylococcus aureus* and in early adulthood most have chronic *Pseudomonas aeruginosa* infection. The number of isolates of filamentous fungi increases with age in CF patients [3]. It has been suggested that the widespread use of broad-spectrum antibiotics has favoured the selection of fungi such as *Aspergillus* spp. [4].

Aspergillus fumigatus is the most common mould reported from the sputa of CF patients [5–7]. It is ubiquitous in the environment; in soil, water and decomposing organic matter. Inhalation of spores into the distal airways can lead to colonisation, saprophytic involvement in those with chronic lung conditions such as chronic obstructive pulmonary disease and CF. In healthy individuals, spores are cleared by the mucociliary escalator and phagocytosed by cells of the innate immune system such as macrophages and neutrophils. However in patients with CF, impaired mucociliary clearance, mucus plugging and the ability of *Aspergillus* spp. to evade or interfere with phagolysosome fusion and complement fixation can lead to locally invasive or disseminated infection [8].

The role of *Aspergillus* spp. in the non-ABPA CF lung is unclear, and its presence in the respiratory secretions of CF patients may be asymptomatic or occur in the context of clinical disease. Here, we review the evidence for filamentous fungi causing respiratory infection and worsening lung disease in CF patients.

2. Fungi and cystic fibrosis

The fungal kingdom comprises over 1,000,000 species. Of these, only a small number of thermotolerant fungi, particularly members of the genera *Aspergillus* spp. and *Penicillium* and some yeasts are able to grow at body temperature and are able to non-invasively colonise the airways. The genus *Aspergillus* spp. alone comprises well over one hundred species and several can cause infections in humans. Invasive aspergillosis and chronic necrotizing pneumonia can occur, in immunocompromised patients but these are thought to be rare in CF. Most attention has focussed on *A. fumigatus* because it is the principal cause of ABPA. *A. fumigatus* contains a number of potent allergens that stimulate the antigen presenting cells and T lymphocytes of the adaptive immune system and cause a spectrum of hypersensitivity diseases including sinusitis, bronchitis and ABPA [8].

3. Allergic bronchopulmonary aspergillosis

ABPA is caused by a hypersensitivity reaction to antigens of *Aspergillus* spp. in individuals with pre-existing lung disease such as asthma and CF. This causes a local inflammatory response which is T helper 2 CD4+ mediated, leading to the production of immunoglobulin (IgG and IgE) specific to *Aspergillus* spp. and the release of cytokines in the airways. Persistent inflammation can result in bronchiectasis and fibrosis. There are different diagnostic criteria for ABPA issued by the CF Trust in the United Kingdom and the CF Foundation in the United States making comparisons between studies from different centres more difficult. CF patients with ABPA tend to have higher rates of AC [8] and poorer lung function parameters compared to those with *A. fumigatus* sensitisation only. Further in-depth discussion of ABPA in CF patients is outside the scope of this review and the interested reader is referred to a recent extensive review on this topic [8].

4. Epidemiology of *Aspergillus fumigatus*

The isolation rate of *Aspergillus* spp. from CF respiratory specimens varies widely from 6 to 58% in the reported literature [3,5,9–18] and this is true for both, adult and paediatric patients, and across different geographical areas of the UK, Europe and North America. There are several possible explanations for this observation. Most importantly, different sputum processing protocols with regards to fungi across laboratories including incubation temperatures, length of culture, media employed and conventional versus molecular detection will influence the detection rate [5,9,10,13,14,17]. Different sampling techniques like cough swabs, spontaneous and induced sputum, and BAL samples may affect *A. fumigatus* detection [3]. We compared differences in *A. fumigatus* isolation between routine cough or sputum samples and BAL. When comparing samples obtained by BAL with sputum or cough swabs in children, Saunders et al. found that *A. fumigatus* was reported from only 19.5% of sputum samples and 2.7% of cough swabs obtained within three months of a positive BAL [3]. This suggests that routine respiratory sampling in children may significantly underestimate AC and this merits validation in a larger study.

Molecular typing of *A. fumigatus* shows that more than one genotype can be present in the sputa of a CF patient [6,19]. Cimon et al. [19] examined serial sputa from CF patients aged 11 to 20 years old and found that those patients who were newly

colonised with *A. fumigatus* harboured several genotypes but eventually one genotype became predominant and was selected over time in those who became chronically colonised. The authors described four patterns of colonisation; (1) identical or related genotypes were isolated over a long period of time indicating that the patients were unable to clear the isolate, (2) one predominant genotype that was not cleared from the airways, (3) a succession of genotypes suggesting that clearance was achieved but re-colonisation occurred from different isolates, and (4) unique genotypes, found only once in a patient, which suggested an ability to clear that isolate. The recurrence of the same genotype over a period of time suggests an inability to clear the fungus from the airways and this may be significant in the pathogenesis of clinical disease.

A retrospective analysis of 1421 patients from 1985 to 2005 performed in adult patients cared for at the Royal Brompton Hospital, London, UK found a significant decrease in AC from 18% to 9% [20]. The authors suggested that different antibiotic practices compared with other centres may account for these findings. In contrast, a recent Canadian CF registry cross-sectional review comparing 3354 adult and paediatric patients in 2005 with 3849 subjects in 2010 showed an increase in the prevalence of *Aspergillus* spp. from 443 (13%) to 785 (20%) [21]. These two studies demonstrate that the difficulties in directly comparing data on *Aspergillus* spp. colonisation and infection of the CF lung as local clinical and microbiological practices and surveillance are likely to be different. Changes in the airway microbiome over time, with *Aspergillus* spp. filling the empty seats, a changing milieu at the level of the airway surface but also frequency, type and administration route of antibiotics and increased exposure over time have all been suggested as potential factors in the increase of *Aspergillus* spp. colonisation with increasing age. It is of course possible that there are genuine differences in the prevalence of fungal colonisation in patients from different geographical areas.

5. Laboratory processing

There are many different methods for the culture of filamentous fungi. In the case of *Aspergillus* spp., these are rapidly growing on almost any media and increasing the incubation time beyond 48 h is unlikely to increase the yield. However, the volume and dilution of sputum affects the sensitivity of fungal detection and prolonging the incubation period for up to four or six weeks increases the recovery of slow growing fungi such as, for example, *Exophiala* spp. [22].

In the United Kingdom there are different national guidelines in existence from the Health Protection Agency (HPA) [22] and the CF Trust [4] for the culture of fungi from respiratory secretions of CF patients. The UK CF Trust only recommends that Sabouraud medium should be incubated up to ten days “at the appropriate temperature”. The HPA advice is to incubate homogenised and diluted whole sputum on Sabouraud agar at 30 and 35 °C, and the plate, to be read at 40 to 48 h with the option of incubation up to six weeks for slow-growing fungi. Whilst this is more specific, this is rarely done in a clinical laboratory setting. In fact, laboratory techniques to identify other filamentous fungi, particularly slow growing ones, are not routinely established and these are therefore rarely reported. New methods to increase sensitivity of fungal culture are being explored. Pashley et al. compared different dilutions of homogenised whole sputum and neat plugs inoculated onto media and found that the rate of detection of *Aspergillus* spp. was dependent on the volume and dilution of sputum used and that the neat sputum plug was better than homogenised diluted or whole sputum [23]. Different media with novel combinations of antimicrobials may be superior in detecting fungi compared to the traditionally used Sabouraud dextrose agar [24]. This is an important area and more research is probably needed before a standard can be recommended. Given these controversies it is not surprising that a recent study by Borman et al. found considerable differences in the processing methods for CF samples in eight microbiology laboratories in England (n=2) and France (n=6) [25]. Laboratories, all of which serve tertiary CF services, differed in the volumes of sputum used for examination, the culture media employed, incubation temperature and length of incubation. Furthermore, variation occurred in laboratories from the same geographical area that served the same cohort of CF patients. The study reported a wide variation in the isolation rates of positive samples for *Aspergillus* spp. from 9 to 89% of patients and surmised that different laboratory processing methods, at least to some extent, are likely to be responsible for the wide variation observed. We recently under took a survey ourselves, of laboratories serving paediatric CF centres in the United Kingdom [Koo et al. 2011 — personal communication]. Between the 19 laboratories that participated in the survey there was variation in the initial processing with lytic agent, volume of sputa used to inoculate plates, type of media, length of incubation and temperature, confirming the findings reported by Borman et al. [25].

6. Antibacterial therapy and *Aspergillus* spp. colonisation

CF patients frequently receive broad-spectrum antibacterials via a variety of routes including oral, intravenous and increasingly nebulised, either as prophylaxis or for the treatment of respiratory exacerbations. Several studies have highlighted the association between nebulised antibacterials and *Aspergillus* spp. isolation from the airway. A large multicentre, double-blind, placebo-controlled trial of intermittent (month on, month off) administration of nebulised tobramycin over a 24-week period, involving 520 CF patients aged six years and older with *P. aeruginosa* infection, found a significantly increased AC rate in the tobramycin treated group [26]. *A. fumigatus* appears rapidly after the initiation of this treatment as it was found in sputum four weeks after the initiation of treatment in a separate tobramycin versus colomycin comparison study [27]. *Aspergillus* spp. were isolated more frequently in the tobramycin treated group (5.7% versus 3.2%). The findings from these large clinical trials are supported by two large retrospective cohort studies [12,18] that investigated the association between AC and inhaled antibacterial therapy. In the largest of these de Vrankijer et al. reviewed the medical records and reports from respiratory samples of 259 children and adults. In a multiple logistic regression model the authors found that AC was independently

associated with inhaled antibiotics and ($P=0.001$) even after correction for severity of lung disease (by correcting for FEV_1) [18]. This was also found in a large cohort of adult CF patients where additionally AC was also associated with the use of prophylactic oral antibiotics [12]. In contrast, a retrospective study in French children found no association between *Aspergillus* spp. isolation from the respiratory tract and inhaled antibacterials [16]. However, it is interesting to note that an increase in airway fungal isolation rates with age has been reported by several studies [10,11,18] which may partly be due to the nature of the respiratory specimens available for analysis. Clearly, the relationship between antibiotic usage and AC is a complex one that requires clarification. The selective pressure of antibacterials may reduce competition and leave an unfilled niche for *Aspergillus* spp. colonisation and infection. The type of antibacterial and the method of administration may have different effects on *Aspergillus* spp. acquisition. From the available evidence, it does appear that inhaled antibiotic therapy, particularly tobramycin, may be associated with an increase in AC. It has to be stressed however that any potential clinical implications of AC are largely unknown and would need to be balanced with the beneficial effects of antibiotic therapy.

7. *Pseudomonas* infection and *Aspergillus* spp. colonisation

Studies investigating the association between *Pseudomonas* and *Aspergillus* spp. colonisation and infection have yielded conflicting results. A recent large retrospective review in Canadian children with CF found a significant link between *P. aeruginosa* and *Aspergillus* spp. respiratory infection [15] but this has not been consistently reported in the literature [12,16–18].

Three studies reported from France [13,17,18] found no significant association between *Pseudomonas* and *Aspergillus* spp. isolation in either children or adults. Paugum et al. reviewed 657 sputum samples from 201 CF adults during a 24-month period and whilst a higher rate of *Pseudomonas* colonisation was present in patients with AC this was not statistically significant (78.9% versus 68.9%, $P=0.07$) [17]. A recent large review of Dutch adults and children with CF found an association between AC and chronic *P. aeruginosa* infection on simple logistic regression analysis that disappeared in a multiple logistic regression model [18]. The authors also performed a longitudinal analysis (over 4 years) splitting the study population into three groups according to the duration of AC. They found no difference in positive cultures for *P. aeruginosa* between the groups. The authors concluded that important confounders may have influenced these results. Patients with longer duration of colonisation were older ($P<0.001$), were more likely to use inhaled antibiotics, ($P<0.001$), had more hospitalisations ($P=0.02$), lower FEV_1 values ($P<0.001$) and lower BMI Z-scores ($P=0.04$). The results of these studies suggest that the relationship between *Pseudomonas* and AC may partly be explained by the age of the study population. Interestingly however, there is in vitro evidence that on a molecular level *P. aeruginosa* produces proteases that directly affect and damage the respiratory epithelium and it has been suggested that this may promote *A. fumigatus* sensitisation [28]. In vitro analysis of culture filtrates of *Aspergillus* spp. from patients with invasive aspergillosis shows a slowing of ciliary beat [28]. Other, secondary metabolites produced by certain Gram-negative bacteria including *Pseudomonas* spp. may change the airway surface milieu and act as fungal growth inhibitors. Pyocyanin, a toxin produced by *P. aeruginosa* has been shown, in vitro, to inhibit growth of *A. fumigatus* and *Candida* spp. in a dose-dependent manner [29]. The in vitro concentrations used were relatively high (pyocyanin minimum inhibitory concentrations for *Candida albicans* and *A. fumigatus* were >64 μg/ml) and it is not clear if such concentrations could be achieved in vivo. The interaction between respiratory pathogens like *P. aeruginosa* and filamentous fungi is likely to be a complex one and this merits further study. Whether the presence of both microorganisms simultaneously has a cumulative effect of damaging respiratory epithelia in CF patients over time remains to be shown.

8. *Aspergillus* spp. colonisation and severity of cystic fibrosis lung disease

The key clinical question of course is whether *Aspergillus* spp. cause respiratory disease in CF in their own right or if filamentous fungi are no more than innocent bystanders. There are no prospective studies to answer this question. A recent large retrospective cohort study in Canadian children reported patient data reviewed over a seven year period [15]. *Aspergillus* spp. infection was defined as two or more positive sputum or BAL samples in one year. Children with persistent *Aspergillus* spp. infection had a lower FEV_1% predicted over the study period compared to uninfected children. In a regression model, children with *Aspergillus* spp. infection had the greatest risk of pulmonary exacerbations requiring hospitalisation (RR = 1.94, $P=0.0002$) which was independent of other factors. There was however a very strong association between *Aspergillus* spp. infection and *Pseudomonas* isolation in the study group.

In a study of 85 French children with a mean age of 8.5 years and a mean observation period of six years [16] the authors found no significant association between AC (defined as two or more positive samples for *Aspergillus* spp. species over a one-year period) and FEV_1% predicted, body mass index or any other measure of disease severity [16]. Similarly no link between AC and lung disease severity was found in a similar study reported by de Vrankijer et al., where AC was defined as the presence of *A. fumigatus* in >50% of respiratory cultures in one calendar year. AC was independently associated with increased age and the use of inhaled antibiotics. Adjusted for confounders like age and BMI Z score AC was not independently associated with lung function decline although the authors report that AC was found predominantly in those with more severe disease and treatment burden [18]. Two older retrospective studies in German [12] and French children [10] found no associations between AC and clinical parameters like FEV_1, Shwachman–Kulczycki (S–K) scores and radiological scores of lung disease.

In contrast to these studies, two reports from US CF centres reported lower S–K and higher chest radiograph scores in patients who had *Aspergillus* spp. isolated from the airways

Table 1
Proposed definitions for *Aspergillus* spp. colonisation and infection.

Aspergillus spp. colonisation	*Aspergillus* spp. infection
• Isolation of *Aspergillus* spp. from 50% or more sputum samples over six months to one year • No deterioration in lung function • No increase in respiratory symptoms like cough	• Isolation of *Aspergillus* spp. from 50% or more sputum samples over six months to one year • Decline in lung function parameters • Respiratory exacerbation with (increased) cough • *Aspergillus* spp. the only organism isolated from repeated sputum samples • No, or incomplete response to a two to four week course of appropriate broad spectrum antibiotics

[9,11]. Both these studies assigned patients to the *Aspergillus* spp. group on the basis of a single isolate which does not constitute colonisation and these studies are not directly comparable to the more recent studies stipulating repeated isolation for a diagnosis of AC.

It is important to highlight that the heterogeneity of study designs with variable duration of study periods, the different techniques of obtaining respiratory samples, varying definitions of *Aspergillus* spp. colonisation and *Aspergillus* spp. infection and different laboratory protocols make comparisons between studies difficult.

All the published observational studies are retrospective and from the available data we conclude that AC has been associated with increasing age, co-infection with *P. aeruginosa* and the prolonged use of oral and/or nebulised antibiotics. All these are important confounders and as a result the isolated contribution of *Aspergillus* spp. colonisation or infection of the airways on progression of CF lung disease remains an unresolved question.

9. The use of antifungal therapy is controversial in non-ABPA CF patients

There is, to our knowledge, only one recently reported Canadian multicentre randomised, placebo-controlled pilot study involving 35 non-ABPA CF adults and children [30]. Patients over six years of age were selected if they were deemed to be *A. fumigatus* colonised but clinically stable and free from acute infection at the time of enrolment. A requirement for study entry was two positive sputum cultures for *A. fumigatus* in the previous year with one positive culture within the last four months preceding enrolment. Subjects were randomised to daily oral itraconazole or placebo for 24 weeks. There was no difference in the rate of exacerbations requiring intravenous antibiotics over the study period between the two groups which was the primary outcome measure. There were also no differences in FEV_1 or quality of life measures between the groups. Adequate plasma levels were achieved in only approximately half the patients. In addition, itraconazole resistance has been demonstrated in vitro for *A. fumigatus* but this was not tested. The study highlights the difficulties encountered when dealing with *A. fumigatus* isolated from CF sputum as it remains uncertain whether such a finding constitutes simple colonisation with no impact on lung disease or true respiratory infection. Based on the reviewed literature we propose the following criteria for *Aspergillus* spp. colonisation and infection which we have summarised in Table 1. Future randomised, placebo-controlled trials based on these considerations and using agents with a better oral bioavailability, such as voriconazole, or a better pharmacokinetic profile would be warranted.

Oral triazoles have been used in the treatment of pulmonary exacerbations and reported in the form of either cohort studies or case series without randomisation, particularly in patients with repeated isolation of *Aspergillus* spp. in the absence of other organisms where repeat courses of broad spectrum antibiotics have failed to resolve the exacerbation. These studies have been summarised in Table 2. Kanthan et al. retrospectively compared two cohorts of children with CF sensitized to *A. fumigatus* over two 5-year periods (1996–2000 and 2001–2005). Those in the latter cohort had higher FEV_1% predicted and this was attributed to them receiving azole treatment more frequently than the earlier cohort. The authors state that there were no significant differences in treatment between the two cohorts with respect to oral or inhaled corticosteroids, azithromycin, nebulised rhDNase

Table 2
Summary of published studies using antifungal medication to treat *Aspergillus* spp. respiratory colonisation or infection in patients with CF.

Study	Patients	Intervention	Outcome
Hilliard et al. [32]	n=21; median 11.3 (5–16) years. Mixture of ABPA and non-ABPA subjects.	All children received voriconazole for a median of 22 (1–50) weeks.	Significant increase in FEV_1 and FVC over the study period in ABPA subjects only.
Shoseyov et al. [33]	n=6; median 14 (10–30) years.	Oral itraconazole for 4 months up to 2 years. One patient also received iv Ambisome.	All experienced some improvement in clinical parameters, FEV_1% predicted improved in all but one.
Kanthan et al. [31]	n=85; *1996 cohort*; *Af* cases, n=19, median age 13.66 (9.78–15.99) years; controls n=19, median age 12.44 (9.02–14.96) years. *2001 cohort*; *Af* cases n=24, median age 12.58 (9.61–16) years; controls n=23, median age 14.61 (12.68–15.29) years.	Observational cross-sectional cohort study using two cohorts of patients (1996–2000 and 2001–2005).	Patients in the 2001 cohort received more antifungal therapy and had a higher FEV1% predicted compared to the 1996 cohort.
Aaron et al. [30]	n=35; enrolled patients 6 years and older, mean age 25 years in both groups.	Oral itraconazole for 24 weeks or placebo.	No difference in exacerbation rates, FEV_1% predicted or QOL between the two groups.

and intravenous antibiotic courses [31]. However this method of comparing cohorts from different time points is problematic particularly as approximately half of the subjects with *A. fumigatus* sensitisation also had ABPA. The authors did not report whether there were any differences in outcome between ABPA and non-ABPA, presumably due to small numbers to begin with. A further retrospective case review of 21 children treated with voriconazole (13 of who fulfilled ABPA criteria) found clinical improvement in patients with ABPA but no significant changes in FEV_1% predicted in those without ABPA. Furthermore, some children who fulfilled ABPA criteria were additionally prescribed immune-modulatory therapy [32]. Finally, a small case series (n = 6) of CF children with respiratory deterioration and positive sputum culture for *A. fumigatus*, who did not fulfil ABPA criteria, reported variable clinical improvement in all patients treated with anti-fungal agents [33]. Treatment was given for up to two years and not all children showed improvements in respiratory function.

9.1. Brief summary of treatment options

Three oral drugs are available with activity against *Aspergillus* spp., itraconazole, voriconazole and posaconazole. These agents vary with regards to their pharmacokinetic and pharmacodynamic properties, side effect profiles, cost and in rates of resistance. Itraconazole and voriconazole have been used more extensively than posaconazole in the CF population. Therapeutic drug monitoring is recommended for all three agents due to poor bioavailabilty with itraconazole and to a lesser extent posaconazole and the highly variable metabolism of voriconazole, especially in children. Resistance to all triazoles is growing but this is especially a problem with itraconazole where >20% resistance has been reported. This may preclude their use in the not too distant future. Intravenous agents available with activity against *Aspergillus* spp. include conventional and lipid formulations (e.g. Ambisome) of amphotericin B and the echinocandins which include caspofungin and micafungin. Ambisome and caspofungin have been used in CF patients post transplant for the treatment of invasive aspergillosis but experience in the treatment of airway colonisation or infection is very limited. Aerosolised amphotericin B and lipid formulation of this agent have been used in adult and paediatric CF patients with ABPA.

10. Conclusion

The contribution of fungal infection to the progressive deterioration of respiratory function in CF is controversial. Whether, or when, isolation of *Aspergillus* spp. from the lower respiratory tract of patients with CF represents harmless colonisation or active infection is not well understood. The evidence so far is largely anecdotal and derived from retrospective, often small case series and does not allow firm conclusions as to whether anti-fungal treatment is justified, and if so, who should be treated and for how long. A recent randomised, placebo controlled trial in CF patients with *A. fumigatus* airway colonisation found no benefits of antifungal treatment with respect to exacerbations or lung function.

The lack of standardisation of laboratory detection methods and differences in the nature of respiratory samples make interpretation of the published data difficult. Antifungal therapy is expensive and not without risks of significant side-effects. The clinical implication of *Aspergillus* spp. isolation from sputum needs to be studied prospectively based on robust national and international protocols for the processing of CF sputum samples for fungi. Finally and importantly there needs to be a consensus on what likely constitutes *Aspergillus* spp. infection warranting specific treatment which would pave the way for an appropriately powered randomised placebo controlled trial in order to conclusively establish the potential benefits of antifungal therapy in such patients.

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