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Review Article

Review Article

The human lung and *Aspergillus:* You are what you breathe in?

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Abstract

The diversity of fungal species comprising the lung mycobiome is a reflection of exposure to environmental and endogenous filamentous fungi and yeasts. Most lung mycobiome studies have been culture-based. A few have utilized next generation sequencing (NGS). Despite the low number of published NGS studies, several themes emerge from the literature: (1) moulds and yeasts are present in the human respiratory tract, even during health; (2) the fungi present in the respiratory tract are highly variable between individuals; and (3) many diseases are accompanied by decreased diversity of fungi in the lungs. Even in patients with the same disease, different patients have been shown to harbor distinct fungal communities. Those fungal species present in any one individual may represent a patient's unique environmental exposure(s), either to species restricted to the indoor environment, for example, *Penicillium*, or species found in the outdoor environment such as *Aspergillus*, wood and vegetation colonizing fungi and plant pathogens. In addition to causing clinical fungal infections, the lung mycobiome may have inflammatory effects that can cause or worsen lung disease. Most respiratory diseases that have been studied, have been associated with decreases in fungal diversity. However, none of these diversity studies distinguish between accidental, transient fungal colonizers and true residents of the respiratory tract. Where does *Aspergillus* feature in the mycobiomes of the respiratory tract? Do these mycobiomes reflect the diversity of fungi in outdoor and internal environments? These intriguing questions are explored here.

Key words: Aspergillus, Mycobiome, Respiratory tract.

Introduction

Many different manifestations of aspergillosis are well understood including allergic aspergillosis (rhinosinusitis and bronchopulmonary) (>10 million worldwide), chronic pulmonary and rhinosinus aspergillosis (~3 million worldwide), invasive aspergillosis (incidence >300000 annually), and superficial disease (notably keratitis, otomycosis, and trauma or burn wound infections). These conditions are seen worldwide.[1]

Allergic bronchopulmonary aspergillosis (ABPA) presents as poorly controlled asthma, production of mucous plugs, and/or mucoid impaction, "pneumonia" and serologically positive in patients with asthma. Poor asthma control in severe asthma is associated with *Aspergillus* sensitization. In cystic fibrosis it presents with pulmonary infiltrates and worsening lung function. Extrinsic allergic bronchoalveolitis may be attributable to *Aspergilli*.

Chronic pulmonary aspergillosis (CPA) usually occurs in patients with underlying pulmonary disease and presents with persistent cough, hemoptysis, weight loss, breathlessness, and fatigue. It may lead to respiratory failure through progressive fibrosis (destroyed lung).

Invasive aspergillosis (IA) occurs in hematological malignancy after transplantation, in those with chronic obstructive pulmonary disease (COPD) and those treated with corticosteroids. IA is now reported in other immunocompromised patient populations, such as intensive care unit (ICU) patients, patients given anti-tumor necrosis factor (anti-TNF) therapy, and patients with AIDS. The early clinical features are usually silent; pyrexia is unusual, and later manifestations include chest discomfort and mild hemoptysis.

Many more fungi are present in outdoor and environments, some of which may impact on human health, beyond the familiar clinical manifestations of aspergillosis. What is not known is whether the presence of other moulds and yeasts in the respiratory tract impact on or exacerbate diseases associated with *Aspergillus*.

Allergic bronchopulmonary mycosis (ABPM) is a hypersensitivity-mediated disease of the lower airways caused by environmental fungi. The predominant etiologic agents include *Candida albicans, Schizophyllum commune*, species of *Alternaria, Bipolaris, Cladosporium, Curvularia, Fusarium, Penicillium, Pseudallescheria, Rhizopus, Saccharomyces, Stemphylium*, and *Trichosporon*.[2] Many of these moulds are found in both outdoor and indoor air.

This review explores the diversity of the mycoflora (the fungal ecosystem) in outdoor and indoor air, and dust from outdoor and indoor environments, and discusses how this matches the mycobiome of the respiratory tract considering how much air is inspired (approximately 40 cubic meters every 24 hours by an adult) and what is the proportion of *Aspergillus* in relation to other environmental moulds. Is *Aspergillus* under- or over-represented in the human respiratory tract? This may help to explain the pathophysiology of pulmonary aspergillosis underlying the relationship between mould pollution of outdoor and indoor environments and these diseases. One major limitation of these studies is that they do not distinguish between transient colonization of the respiratory tract or resident flora. An analogous scenario is the gut mycobiome. There appears to be an impressive diversity in the gut but one view is that this is illusory.[3] There is a clear division between a small number of commonly detected species (*Candida, Saccharomyces*, yeasts in the Dipodascaceae, and *Malassezia* species) and a long list of taxa that have been reported only once. Furthermore, an investigation into the ecology of these rare species reveals that many of them are incapable of colonization or long-term persistence in the gut. This thinking should be kept in mind when reviewing the long list of taxa in the respiratory tract.

There are a number of challenges regarding mycobiome research.[4] First, the isolation of nucleic acids from fungal cells can be problematic and often requires a combination of enzymatic, chemical, and mechanical lysis steps. Second, the ability to discriminate between fungal taxa is influenced by sequencing primer choice; finally, curated databases for taxonomic assignment and/or the annotation of fungal genomes are lacking, are incomplete, or inaccurate.

Perhaps the major challenge when reviewing the literature on the mycobiome of the respiratory tract is an understanding of the nuances of the various techniques used and how divergent results have been generated, that is, culture versus nonculture methodologies, and the various nonculture molecular platforms. This is beyond the scope of this review. The reader is directed toward the numerous analyses of the gut mycoflora that have demonstrated the efficiency of these techniques in identifying a high number of fungi but also have revealed major discrepancies between surveys.[5,6] Culturomics has emerged recently as a successful tool to isolate high number of bacteria from the gut and to identify new species.[5] It has shifted the view on human gut microbiota by discovering "new" bacterial diversity not previously captured by metagenomics. However, culturomic methodology has not yet been comprehensively applied to describe the fungal population in the human gut or the respiratory tract.

It is against this background and understood limitations of the methods used that the various surveys of the fungal diversity within the respiratory tract are discussed.

The mycobiome of outdoor air and dust

While the physical and chemical properties of particulate matter (PM) pollutants have been extensively studied, relatively little is known about microbes associated with PM, especially airborne fungi.[7] Fungal spores can account for large proportions of air PM, and some fungi are major pathogens or allergens for humans. Moreover, air is the primary medium for fungal dispersal.[8] Hitherto, a few studies carried out provide only limited insight into airborne fungi associated with PMs. Airborne fungi associated with PMs have been reported from different regions of the world.[7] A few examples are given here.

To assess the diversity and composition of airborne fungi associated with PM in Beijing, China, a total of 81 PM samples were collected, which were derived from PM2.5, PM10 fractions, and total suspended particles during hazy and non-hazy days.[7] The airborne fungal community in these samples was analyzed using the Illumina Miseq platform with fungi-specific primers targeting the internal transcribed spacer1 region of the large subunit rRNA gene. A total of 797040 reads belonging to 1633 operational taxonomic units were observed. Of these, 1102 belonged to Ascomycota, 502 to Basidiomycota, 24 to Zygomycota, and five to Chytridiomycota. The dominant genera were *Cladosporium*, *Alternaria*, *Fusarium*, *Penicillium*, and *Aspergillus*. Analysis of similarities revealed that both particulate matter sizes and air quality levels significantly affected the airborne fungal community composition. The relative abundance of many fungal genera was found to significantly differ among various PM types and air quality levels. *Alternaria* and *Epicoccum*

were more abundant in total suspended particle samples, *Aspergillus* in heavy-hazy days and PM2.5 samples, and *Malassezia* in PM2.5 samples and heavy-hazy days. Furthermore, temperature and relative humidity were significant factors that determined the composition of the airborne fungal community. The results suggest that diverse airborne fungal communities are associated with particulate matters.

Numerous reports have been published on the association between the airborne *Aspergillus* conidia and fine particulates, while limited studies have focused specifically on the association between invasive aspergillosis and ambient fine particulate air pollution. Liu and colleagues investigated in Taiwan the association between ambient fine particulate PM2.5 air pollution and invasive aspergillosis at a population level.[9] They had access to a collection of long-term and nationwide databases of daily ambient PM2.5 levels and invasive aspergillosis incidence. A total of 1000000 patients during the study period (1999–2009) were included in the data set. The findings suggested a positive association between PM2.5 concentration and incidence of aspergillosis. Furthermore, month-wise invasive aspergillosis case numbers potentially demonstrated lagged pattern following peaking of PM2.5 concentration, a delay between PM2.5 exposure and invasive aspergillosis, of approximately 1 month was seen.

The concentration of fungal species and particles (PM10 and PM2.5) has been investigated in two French hospitals, as well as ambient parameters (temperature, relative humidity, pressure, and carbon dioxide).[10] Chemical and microbiological air concentrations were measured during two campaigns (winter and summer) and across seven rooms. The study showed that indoor air contains a complex mixture of chemical and physical compounds and a variety of fungi: *Aspergillus, Penicillium, Alternaria, Cladosporium,* and *Stachybotrys*. Concentrations in the same order of magnitude were found in both hospitals.

Fungal communities: indoor dust and air—houses

House dust is a complex mixture of inorganic and organic material that supports a diverse population of microbes, reviewed by Rintala and colleagues.[11] Filamentous moulds and yeasts are able to grow and proliferate in dust only if there is enough moisture present. Most of the fungal population originates from sources other than the dust itself, these being outdoor air and other outdoor material, such as vegetation or dirt brought into buildings, occupants of the buildings themselves, including pets and fungal growth on moist building materials.

Based on numerous culture and nonculture based studies, *Penicillium, Aspergillus, Cladosporium*, and about 20 other fungal genera are the most commonly found in dust depending on the dust type, detection method, type of the indoor environment and season, among other factors.[11,12] Microbial assemblages in different house dust types usually share the same dominant species; however, alterations in the composition are caused by differing sources of microbes for different dust types. For example, mattress or pillow dust is dominated by species originating from the user of the mattress, and temperate climate zones show higher dust microbial diversity than tropical zones.

The house dust components of fungal origin may include intact fungal conidia, spores, and spore clumps, as well as fragments of spores and hyphae. The size and shape of intact fungal spores varies from tiny, round to ovoid 2–5 μm conidia of *Aspergillus* and *Penicillium*, to large, oblong 50 μm conidia of *Alternaria* and *Helminthosporium*. Fungal fragments vary in size from submicrometer (<1 μm) particles to larger hyphal fragments.

The indoor fungal ecosystem is complex and is thought to depend on dispersal from the outdoor ecosystem and the occupants' mycobiome combined with selective pressures imposed by the occupants' behaviors and the building itself. Early studies explored the occurrence of fungi in pillows, furniture stuffings, and upholstery as well as house dust in relation to asthmatic reactions and allergic sensitization.[11] The first studies to explore dusts collected from indoor environments reported the basic findings concerning levels of viable fungi, the prevalent indoor and outdoor taxa, the strong variation in fungal taxa in indoor air, and the tendency of indoor materials to accumulate fungal spores over time that have since been verified by numerous other studies.[11]

Fungal species capable of proliferating in house in the absence of moisture include extreme xerophiles: for example, *Eurotium repens*, *Aspergillus penicilloides* and mixed *Penicillium* species. Most other moulds, for example, *Cladosporium* and *Alternaria,* are considered to be unable to proliferate under dry indoor conditions but need a liquid water source or excessive indoor humidity.[13] These two species are commonly found in outdoor air in a number of global regions.

The investigation of indoor microbial ecology has been based primarily on culture, but more recently it has been facilitated by nucleic acid sequence-based methods, reviewed by Adams and colleagues.[14] Together, these approaches have identified fungi that grow indoors when moisture is available that are shed from humans or pets[15] and those species that have obvious outdoor origins.[16] From recent studies that have relied on nucleic acid amplification (NAAT) techniques to assess total fungal assemblages it is clear that fungi in settled dust are diverse, that variation between buildings can be large and fluctuate with the outdoor air, and that, as a result, there may not be a typical profile of indoor fungi that distinguishes different types of buildings.[17] What are the processes that influence the dispersal and selection regimes of moulds in the indoor space? First, it is clear that the majority of indoor air fungi are derived from outdoor air.[11] Second, the variation in fungal communities between houses, for example, is large and partly attributable to selection pressures imposed by resident behavior. A third finding is that airborne fungal communities vary among rooms within a building based on room use

and water availability. Furthermore, it is expected that cleaning and cooking patterns and exposure to the outdoors will have a predictable effect on the composition of the indoor fungal communities and that the air in rooms with water sources (bathrooms and kitchens) would have a higher burden of resident fungi.

Numerous studies have determined the pattern of fungal diversity and composition in indoor air in specific in indoor environments in order to identify processes supporting the array of species. Adams and colleagues surveyed airborne fungal assemblages within 1-month time periods at two seasons, indoors and outdoors, within and across residences at a university housing facility.[14] Fungal communities indoors were diverse and strongly determined by dispersal from outdoors, and no fungal taxa were found as indicators of indoor air. There was a seasonal effect on the fungi found in both indoor and outdoor air, and quantitatively more fungal biomass was detected outdoors than indoors. Interestingly, room and occupant behavior had no detectable effect on the fungi found in indoor air. These results confirm previous reports that outdoor air fungi dominate the spectrum of indoor air. More broadly, this study provides additional support for the growing evidence that short-distance dispersal, even on small geographic scales, is a key process in structuring the often-observed distance–decay biogeographic pattern in fungal communities.

To determine the diversity of moulds that residential occupants are exposed Tong and colleagues observed that common skin and environmental fungal taxa dominated air, surface, and skin samples in residences in Hong Kong.[18] The frequency of how often individuals touch surfaces in their homes appeared to determine the fungal community structure on occupant residential surfaces. SourceTracker prediction suggested that some fungi can be transferred bi-directionally between surfaces and skin sites (SourceTracker software is designed to predict the source of microbial communities in a set of samples). In addition, the study demonstrated a modest but significant association between indoor airborne bacterial composition and geographic distance on a city-wide scale but not for fungi. It was clear that airflow might play a prominent role in driving the spatial variation of the indoor airborne mycobiome. This and local environmental factors, including air currents, appear to be stronger determinants of indoor airborne mycobiome than ventilation strategy, human occupancy, and room type.

Little is known about the indoor fungal ecosystem of green homes. Green housing is designed to use low-impact materials, increase energy efficiency, and improve occupant health. A recent study looked at the variability of fungal populations in green and nongreen homes.[19] The fungal diversity in air, bed dust and floor dust was compared between green and nongreen, low-income homes in Cincinnati, Ohio, USA. The samples were collected at baseline (within four months following renovation), and 12 months after the baseline in the green homes. Parallel sample collection was conducted in nongreen homes. Air samples were collected by PM2.5 samplers over 5 days. Bed and floor dust samples were vacuumed after the air sampling was completed. The DNA sample extracts were analyzed using ITS amplicon sequencing. Analysis indicated that there was no clear trend in the fungal communities between green and nongreen homes. Instead, fungal community differences were greatest between sample types: air, bed, and floor. Fungal communities also changed substantially between sampling intervals in both green and nongreen homes for all sample types, potentially indicating that there was very little stability in the fungal ecosystem.

The fungal composition of household dust has been analyzed using quantitative polymerase chain reaction (MSQPCR) technology developed by the US Environmental Protection Agency.[20] The presence of 81 mould species in homes in the United States and the United Kingdom was determined. Dust samples were obtained from randomly selected homes in the United Kingdom (11 homes). The mould populations in British homes were compared with those found in typical homes (no visible mould) in the United States (in the state of Ohio, 45 homes). Only 13 of 81 species screened showed significantly different concentrations in these two sets of homes. Although only a small survey, the results suggested that typical mould profiles in the United States (Ohio) and British homes were very similar. Analysis of 26 mould indicator species revealed that the British homes fell into two clusters, tentatively identified as "atypical" and "typical" mould conditions. This study was an early demonstration that nucleic analysis amplification of dust samples could provide an objective measure of indoor moulds and the potential for exposure and associated health effects.

The scale of this approach to explore the fungal communities in homes has been extended to other parts of the globe.[21] As part of a worldwide survey of the indoor mycobiota, dust was collected from nine countries. Analyses of dust samples included culture and pyrosequencing. A total 7904 isolates were recovered; of these, 2717 isolates were identified as belonging to *Aspergillus, Penicillium,* and *Talaromyces* groups. In addition, the study created a reliable reference sequence database for next-generation sequencing projects. Fifty-nine *Aspergillus* species were identified, including eight undescribed species, 49 *Penicillium* species of which seven were undescribed and 18 *Talaromyces* species including three new ones. In total, 568 ITS barcodes were generated, and 391 β-tubulin and 507 calmodulin sequences, creating a database of alternative identification markers.

A number of studies suggest that a big proportion of yeasts in house dust originate from human skin, particularly notable during months where the ingress of outdoor sources is reduced. For example, Pitkaranta and colleagues showed that a significant proportion of fungal diversity in house dust of two nursing home buildings in Finland during winter months originated from the basidiomycete subclass *Exobasidiomycetidae*, consisting mainly

of *Malassezia*.[22] Generally, winter samples were dominated by yeasts, while all other seasons were dominated by filamentous fungi.

Fungal ecosystem: indoor air and dust—hospitals

Fungal contamination in healthcare facilities has been the subject of numerous studies that have explored transmission of airborne spores through ventilation systems and air conditioning units. Few studies have attempted to correlate the level of fungal pollution with the occurrence of specific diseases among patients or hospital staff. However, these studies do provide information on the diversity of moulds in indoor air and what patients and staff are exposed to. The degree of contamination by fungi in the hospital environment may increase dramatically in combination with various factors, such as the presence of construction activity, demolition, and a favorable microclimate. Because exposure to fungi can cause serious health problems, it is clearly essential, in the above-mentioned risk situations, to evaluate the degree of contamination in the various environments and to use those evaluations to determine the risk of infection for patients and staff alike. It is clear that the use of air handling systems with in-line filters and the use of air conditioning systems does not provide complete protection against fungi.

Airborne fungi in hospitals are associated with hospital-acquired infections. The high diversity of airborne fungi in the hospital environment has been demonstrated by culture-based techniques and sequencing-based mycobiome analysis. To identify the causative airborne microorganisms, Tong and colleagues used high-volume air samplers for recovering fungi from hospital air.[23] Species identification was performed by culture-based methods and DNA sequencing analysis with the Illumina MiSeq and HiSeq 2000 sequencing systems. The distribution characteristics of fungi were investigated using heat map analysis of four departments, including the respiratory intensive care unit, intensive care unit, emergency room, and outpatient department. The prevalence of *Aspergillus* species was approximately 17% to 61%, and the proportion of *Aspergillus fumi gatus* among *Aspergillus* species was from 34% to 50% in the four departments.

Aspergillus as a member of the mycobiome of the respiratory tract

Molecular techniques have revealed a previously unappreciated complexity to the mycobiome of the respiratory tract that has renewed an interest in the interactions between host, fungi, and the pathogenesis of exacerbations of chronic lung disease. The composition of the lung microbiome is controlled by fungal immigration (i.e., inhalation), elimination, and relative growth rates of the constituent species, reviewed by Dickson and colleagues.[24] All these factors change dramatically in chronic lung disease and further during exacerbations. The features of exacerbations include increased fungal burden and decreased diversity of fungal communities. It has been proposed that exacerbations are occasions of respiratory tract dysbiosis—a disorder of the respiratory tract fungal ecosystem with negative effects on host biology and physiology.[24] Respiratory tract dysbiosis provokes a dysregulated host immune response, which in turn alters the growth conditions for microorganisms in airways, promoting further dysbiosis and perpetuating a cycle of inflammation and disordered microbiota. Differences in the composition of baseline respiratory tract microbiota might help to explain the so-called frequent-exacerbator phenotype observed in several disease states, and might provide novel targets for therapeutic intervention.[24]

To understand how successful has the immigration of *Aspergillus* conidia into the upper and lower respiratory tract the mycobiome of individual compartments and structures will be examined.

The fungal mycobiome in sinusitis

The upper airways or upper respiratory tract includes the nose and nasal passages, paranasal sinuses, the pharynx, and the portion of the larynx above the vocal folds (cords). The lower airways or lower respiratory tract includes the portion of the larynx below the vocal folds, trachea, bronchi, and bronchioles. It has been known for a long time that the membranes of the nasal mucosa are home to a large number of fungi that are found in individuals both with and without sinus disease. A well-described mucosal disease in this region is chronic rhinosinusitis. This is regarded as a disease of inflammation rather than infection. However, numerous studies have demonstrated roles for commensal resident fungi, or their secretory products such as allergens and secondary metabolites, in the initiation and/or progression of mucosal inflammation.

Fungal associated infections of the upper respiratory tract are biofilm diseases. A biofilm is a complex polymicrobial community of fungi surrounded by an exopolysaccharide matrix produced by fungi. Formation of biofilms is a defense mechanism for fungi that can protect themselves from antifungals and from the host immune system. Biofilm formation in sinonasal mucosa has been studied in chronic rhinosinusitis syndrome (CRS) in multiple studies. It is associated with recurrence of disease, poor response to treatment, and unfavorable outcome after surgery. Diverse fungal biofilms have been attributed to the pathogenesis and phenotypes of CRS.[25]

The association of fungi with CRS has been investigated in multiple studies, with controversial results. In the late 1990s, fungal cultures identified colonization in more than 90% of CRS patients and drew significant attention to this field. In this way, several fungal species were identified in CRS.[26] Further studies using nucleic acid amplification techniques showed evidence of

fungal colonization in the nose in more than 90% of healthy controls.[27]

Does *Aspergillus* cause chronic rhinosinusitis? Recent studies that used specific primers for PCR analyses of fungi give a more complete picture of the fungal mycobiome in the nose and paranasal sinuses. However, the results of these studies have engendered controversies as well. Cleland and colleagues analyzed nasal swabs of 23 chronic rhinosinusitis (CRS) patients and 11 controls.[28] They used 18S ribosomal DNA (rDNA) fungal tag-encoded FLX amplicon pyrosequencing. This showed that CRS cases had greater fungal richness than controls. *Malassezia* was the most common and abundant species and was found in all CRS cases at surgery. *Aspergillus* was found in 365 of the patients. Aurora and coresearchers analyzed the mycobiome the sinuses by deep sequencing of 18S rDNA.[29] They found qualitatively similar mycobiomes in CRS and controls but greater diversity and abundance of fungi in the CRS group. *Cryptococcus neoformans* was the most abundant fungus in both CRS and control cases, although it was more prevalent in the CRS group (90% vs 61%). Boase and colleagues focused on pathogenic taxa using 16 primer pairs for PCR, which could survey nearly all pathogenic fungal species, but could not find evidence of pathogenic fungal DNA in most of the cases studied.[30] Three CRS patients with nasal polyps cases were positive (two for *Aspergillus fumigatus* and one for *Bipolaris papendorfii*), while all CRSsNP cases and healthy controls were negative.

A seminal study by Burzina and colleagues examined the biodiversity of fungi isolated from the nasal mucus of patients suffering from chronic rhinosinusitis and from healthy persons, monitored over 28 months by culture and PCR and sequencing.[27] Patients were primarily from the southern province of Styria in Austria. Mucus samples were obtained by flushing the noses of patients with saline or by endoscopic sinus surgery. Altogether, 619 strains of fungi were cultivated from 233 subjects. Eighty-one species were identified, with a maximum of nine different species per person. The most prevalent species were *Penicillium, Aspergillus, Cladosporium, Alternaria,* and *Aureobasidium*. Whereas *Aspergillus* and *Penicillium* spp. occurred in more or less the same numbers throughout the year, *Cladosporium* spp., *Alternaria* spp. and *Aureobasidium pullulans* showed a significantly higher occurrence during late summer and early autumn.

Mycobiome of the oral cavity

The concept of a "core healthy oral mycobiome" was introduced in 2010 by Ghannoum and colleagues when they characterized the oral mycobiome of 20 healthy adults.[31] Analysis of ITS1F/ITS2 sequences identified a total of 85 fungal genera within the oral cavity, 11 of which related to non-culturable fungal genera. Although the exact number of fungal genera in the oral cavity varied between participants (range 5–39), a core set of genera were identified in the oral cavities of more than 20% of study participants: *Candida* (75%); *Cladosporium* (60%); *Aureobasidium* (50%); *Aspergillus* (35%); *Fusarium* (30%), and *Cryptococcus* (20%). The high prevalence of *Candida* in the oral cavity is consistent with previous culture-based studies, and subsequent molecular studies confirmed the high prevalence of *Candida* spp. within the oral cavity.

Another study found that when the oral fungal microbiome was investigated in 30 individuals with and without periodontitis, moulds were recovered from all participants and yeast from 92.5%.[32] The most frequently isolated fungi were *Candida, Rhodotorula, Penicillium, Aspergillus*, and *Cladosporium*.

The mycobiome of the oral cavity of individuals with and without human immunodeficiency virus (HIV) has been analyzed.[33] Although both *Candida* and *Penicillium* were isolated from the oral cavity of all individuals, significant differences in the overall mycobiome profiles were identified between healthy and disease states. For example, *Alternaria, Epicoccum,* and *Trichosporon* were found only in HIV positive patients, while *Pichia, Cladosporium,* and *Fusarium* were associated with health.

The mycobiome of the respiratory tract in the context of specific diseases

The recent application of mass sequencing techniques with high-performance platforms and amplification of total DNA in the respiratory secretions of patients with chronic bronchopulmonary disease has made it possible to detect a wider range of microorganisms than was the case with conventional cultures. Differences have been observed between the yeast identified in healthy individuals and those detected in patients with bronchiectasis or asthma, suggesting that they could have a different significance in different contexts. Most of the research undertaken on the microbiome in respiratory disease has involved small sample sizes, as well as sputum samples (rather than bronchoaspiration), and as a result, its clinical importance is still unclear. The main focus has been on the bacterial microbiome in cystic fibrosis (CF), asthma, and COPD, with only a few studies of bronchiectasis. In the case of CF, fungi are detected more often during exacerbations.

Apart from the infections that are already known to be caused by fungi, the lung mycobiome can have inflammatory effects that can cause or aggravate chronic respiratory disease.[34] Like bacteria, fungi also contain pathogen-associated molecular patterns (PAMP), which are recognizable by pattern recognition receptors that activate macrophages, B and T cells, thereby triggering inflammation. An understanding of the innate and inflammatory consequences of exposure to *Aspergillus* species is critical in accounting for disease manifestations and preventing sequelae. The major components of the innate immune system involved in recognition and removal of the fungus include phagocytosis,

antimicrobial peptide production, and recognition by pattern recognition receptors. The cytokine response is also critical facilitating cell-to-cell communication and promoting the initiation, maintenance, and resolution of the host response.[35] Given that fungi are omnipresent in the environment, the respiratory system's continuous exposure to them and their capacity to trigger an inflammatory process suggests that the mycobiome may contribute to lung damage. Most studies have found that a reduction in fungal diversity in respiratory diseases correlates with a poorer lung function. This reduced diversity could be caused by the excessive growth of one fungal species or by the elimination of other species.

Cystic fibrosis

The mycobiome of the respiratory tract of CF patients is complex. Botterel and colleagues compared the fungal diversity of sputa from adult CF patients during non-exacerbation period by culture-based and molecular methods, and ultra-deep-sequencing (UDS).[36] Sputum samples from four CF patients were cultured and analyzed by DNA extractions followed by terminal restriction fragment length polymorphism analysis through resolution of bacterial ribosomal gene (rDNA) fragments, and cloning plus sequencing of part of fungal rRNA genes. These approaches were compared with the UDS method targeting 16S rDNA gene and the internal transcribed spacer (ITS) 2 region of rDNA. A total of 18 fungal genera were detected from the four patients. The mean number of genera detected by UDS per patient was statistically higher than by culture or whole genome sequencing (WGS) methods. Patients with severe airway disease as assessed by standard spirometry exhibited a reduced fungal and bacterial diversity. UDS approach evaluates more extensively the diversity of fungal and bacterial flora compared with cultures. The authors concluded that their study, despite the small number of enrolled patients, highlighted benefits of UDS for fungal detection. Using the UDS method, they were able to detect taxa not revealed by culture or WGS, at relatively high proportions, and to designate a mycobiota core composed of *Aspergillus* belonging to the Fumigati section and *Penicillium* spp., which was consistent with a previous history of *A. fumigatus* colonization. Similar to other analyses the study showed a reduced diversity of fungal and communities in patients with impaired lung function (low FEV1 and FCV).[36]

Chronic obstructive pulmonary disease

Exacerbations of COPD are associated with high mortality, rapid decrease in lung function, and increased health-care costs. Frequency of exacerbations increases with severity of airway obstruction, but many patients experience exacerbations more frequently than would be predicted by disease severity alone (the so-called frequent exacerbator phenotype).[24] Exacerbations are also associated with systemic inflammation, airway inflammation, and increased airway obstruction due to edema, increased sputum production, and bronchoconstriction.[24] The primary fungus enriched in the lungs of individuals with COPD in the setting of HIV is *Pneumocystis*.[33] *Pneumocystis* colonization has been postulated to contribute to COPD by stimulating inflammation and release of matrix metalloproteases, which can damage the lung.[33]

COPD is present in 2% of patients dying from invasive pulmonary fungal disease. A second presentation in COPD patients is chronic pulmonary aspergillosis. A third presentation is aspergilloma. It is usually asymptomatic although fatal hemoptysis and/or invasion of pulmonary tissue can develop. Finally, patients who are admitted to the ICU with an exacerbation of their disease, can develop invasive aspergillosis with almost 100% mortality rate (reviewed in Ostrosky-Zeichner and Al-Obaaidi[37]).

Asthma

Exacerbations cause much of the mortality, morbidity, and health-care expenses of asthma. Precipitants include allergens, air pollution, and exercise, although, as is the case in COPD, some patients exhibit a frequent exacerbator phenotype independent of other risk factors. In addition to viral infections asthma exacerbations are strongly associated with fungal exposure and fungal sensitization.

Little is known about the mycobiome in fungal lung disease. Fraczek and colleagues determined the mycobiome in lungs of individuals with well-characterized fungal disease and looked at the association with corticosteroid therapy.[38] Following bronchoscopy, ribosomal internal transcribed spacer region 1 DNA was amplified and sequenced and fungal load determined by real-time PCR. Patients with bronchopulmonary aspergillosis, severe asthma with fungal sensitization, severe asthma not sensitized to fungi, mild asthma patients, and 10 healthy control subjects were included in the study. The mycobiome was highly varied with severe asthmatics carrying higher loads of fungus. Healthy individuals had low fungal loads, mostly poorly characterized Malasezziales. Individuals receiving steroid therapy had significantly higher levels of *Aspergillus* and total fungus in their bronchoalveolar lavage.

Bronchiectasis

Little is known about the epidemiology and fungal biodiversity of bronchiectasis.[34] In bronchiectasis patients, deteriorated mucociliary clearance, generally due to prior colonization by bacterial pathogens, and thick mucus accumulation, the persistence of fungal spores in the respiratory tract. The most prevalent fungi in these patients are *Candida albicans* and *Aspergillus fumigatus* (Table 1); these are almost always isolated with bacterial pathogens like *Haemophillus influenzae* and *Pseudomonas aeruginosa*, making it very difficult to define their clinical significance. Analysis of the mycobiome enables us to detect a

Table 1. Species most frequently found in patients with bronchiectasis not associated with cystic fibrosis.

Yeasts	Filamentous fungi
Candida albicans	*Aspergillus fumigatus*
Candida glabrata	*Aspergillus niger*
Candida parapsilosis	*Aspergillus terreus*
Saccharomyces cerevisiae	*Scedosporium apiospermum*
Trichosporon beigellii	*Penicillium* species
Exophiala dermatitidis	*Fusarium* species

(Adapted from Máiz et al.[34]).

greater diversity of microorganisms than with conventional cultures. The results have shown a reduced fungal diversity in most chronic respiratory diseases, and that this finding correlates with poorer lung function.

Aspergillus spp. are the most prevalent filamentous fungi found in bronchiectasis patients.[34] *A. fumigatus* is the most common species, followed by *A. niger*, *A. terreus*, and *A. flavus*. However, there are huge variations in the prevalence of the fungi isolated in studies. The prevalence of *Aspergillus* spp. in bronchiectasis ranges from 7% to 24%. The distribution of the species in bronchiectasis patients varies according to the geographical area, with a high prevalence of *A. niger* and *A. terreus* in Japan and of *A. flavus* in India and China.

The clinical significance of fungi in cultures of respiratory samples taken from bronchiectasis patients has still not been clearly defined. Further studies that would make it possible to standardize and evaluate both the microbiological criteria for defining chronic colonization and the methods used for culturing and identifying fungi would provide us with more precise knowledge of the genera and species involved, and of the role that fungi play in the clinical evolution of bronchiectasis patients.

Very recently, understanding the composition and clinical importance of the fungal mycobiome in patients has been identified as a key topic in a "research priorities" consensus statement for bronchiectasis.[39] As part of the CAMEB study (a multicenter cross-sectional Cohort of Asian and Matched European Bronchiectasis patients) the respiratory mycobiome was determined in 238 patients by sequencing the 18S-28S rRNA internally transcribed spacer regions ITS1 and ITS2. Specific qPCR for detection of and conidial quantification for a range of airway *Aspergillus* species was performed. The mycobiome of patients with bronchiectasis was characterized by specific fungal genera including *Aspergillus*, *Cryptococcus*, and *Clavispora*. *A. fumigatus* (in Singapore/Kuala Lumpur, Malaysia) and *A. terreus* (in Dundee, UK) dominated the mycobiome profiles. Interestingly, *A. terreus* was associated with exacerbations. High frequencies of *Aspergillus*-associated disease including sensitization and allergic bronchopulmonary aspergillosis were detected. Each revealed distinct mycobiome profiles and associated with more severe disease, poorer pulmonary function and increased exacerbations. This study concluded that the pulmonary mycobiome is of clinical relevance in bronchiectasis and the authors advocate that screening for *Aspergillus*-associated disease should be considered even in apparently stable patients.

Lung transplant recipients

Long-term survival after lung transplantation is limited by infectious complications and by bronchiolitis obliterans syndrome (BOS), a form of chronic rejection linked in part to microbial triggers including fungi. Charlson and colleagues defined microbial populations in the respiratory tract of transplant patients comprehensively using unbiased high-density sequencing.[40] Lungs were sampled by bronchoscopy and the upper respiratory tract by oropharyngeal wash. Fungal internal transcribed spacer sequencing was used to profile organisms present. Fungal populations were typically dominated by *Candida* in both sites or by *Aspergillus* in bronchoalveolar lavage fluid but not in oropharyngeal wash. This study demonstrated that the fungal communities in lung transplant recipients differ in structure and composition from healthy subjects. This understanding is of paramount importance when assessing the significance of *Aspergillus* in the cystic fibrosis setting.

What do we breathe in?

Colonization by environmental fungi, including *Aspergillus* may become significant, and eventually result in *Aspergillus*-related lung disease when the microbiological composition of the normal flora in the respiratory tract is disturbed, such as in the case of an underlying disease, an altered immunological status of the host, or a decrease in ciliary activity of the mucous pulmonary epithelium. The underlying diseases that predispose to aspergillosis could thus provide significant insights into which alterations in the microbiome might predispose to aspergillosis. The fungi that reside in the human lungs represent an understudied but medically relevant community. From the few studies published on the lung mycobiome, it is clear that there are fungi in both the healthy and diseased respiratory tract, that these fungi vary widely between individuals, and that there is a trend toward lower fungal diversity among individuals with chronic or progressive respiratory disease. Another dimension to the above discussion is the persistence or clearance of fungal conidia and hyphal fragments in healthy or debilitated individuals from the various levels of the respiratory tract.[41] The primary route of human infection is via the inhalation of these airborne conidia, followed by conidial deposition in the bronchioles or alveolar spaces. In healthy individuals, conidia that are not removed by mucociliary clearance encounter epithelial cells or alveolar macrophages, the primary resident phagocytes of the lung. Alveolar macrophages are primarily responsible for the phagocytosis and killing of *Aspergillus* conidia as well as the initiation of a proinflammatory response that recruits neutrophils (one type of

polymorphonuclear cell [PMN]) to the site of infection. Conidia that evade macrophage killing and germinate become the target of infiltrating neutrophils that are able to destroy hyphae. The risk of developing IA results primarily from a dysfunction in these host defenses in combination with fungal attributes that permit *A. fumigatus* survival and growth in this pulmonary environment.

It is patently clear from the surveys reviewed that healthy individuals and patients are exposed to a diverse collection of fungal taxa and species in outdoor and indoor environments. In many geographical areas *A. fumigatus* is able to grow and sporulate on a variety of organic substrates in outdoor niches and release conidia into the air. Mycobiome studies of air and dust demonstrate this very clearly. The appearance of *Aspergillus* in indoor locations is most likely as a result of migration of conidia from outside considering that there are few substrates and microenvironments to support the growth of *Aspergillus* in homes. The interesting question is whether the lung mycobiome reflects the diversity of fungi in outdoor and indoor environments. It is suggested, based on the limited analysis of the lung mycobiome, reviewed here, that the answer is no. *Aspergillus* species are underrepresented in many respiratory tract mycobiome analyses, but this appears to be highly dependent on the analysis platform used.

Molecular analysis of mucosal surfaces and respiratory secretions from the respiratory tract has shown that fungal respiratory communities are far more complex than culture alone would suggest. For example, analysis of sputum samples from individuals with and without cystic fibrosis demonstrated that over 60% of organisms identified by sequencing were not recovered by culture, although it should be noted that culture techniques for respiratory samples are from optimal. Current national and international standards are woefully inadequate. Individuals with decreased lung function (as measured by FEV1 and FVC) harbour less diverse fungal and bacterial communities, which tend to be dominated by only a small number of organisms. Interestingly, bacterial communities with high abundances of *Pseudomonas* are more likely to be associated with high abundances of *Candida* species than with *Aspergillus fumigatus*. A similar phenomenon has been seen in lung transplant patients, in which *Aspergillus* species were never isolated in individuals with bacterial communities dominated by *Pseudomonas*.

There is a pronounced decreased diversity of fungal communities with the progression of chronic lung disease and exacerbations. It is proposed that exacerbations provoked by fungal exposure are occasions of respiratory tract dysbiosis—a disorder of the respiratory tract fungal ecosystem with negative effects on host biology. Respiratory tract dysbiosis provokes a dysregulated host immune response, which in turn alters growth conditions for fungi in airways, promoting further dysbiosis and perpetuating a cycle of inflammation and disordered mycobiota: a perfect environment for aspergillosis.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and the writing of the paper.

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