Analysis of the fungal microbiome in exhaled breath condensate of patients with asthma

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ABSTRACT

Background: *The presence of virus and bacteria in the airways of subjects with asthma is common and seems to be associated with a deterioration due to the disease. The microbiologic study of airways in asthma is foreseen by guidelines with induced sputum that is often ineffective and contraindicated in severe asthma.*

Aim: *To analyze the fungal microbiome in the exhaled breath condensate (EBC) of subjects with asthma by evaluating a possible correlation with anthropometric and asthma severity data.*

Methods: *We enrolled 47 consecutive subjects with asthma (28 with atopic asthma and 19 with nonatopic asthma) and 20 controls. Enrolled subjects underwent EBC and sputum collection. Fungal microbiome was assessed by culture on EBC and sputum samples by using Czapek yeast extract agar.*

Results: *A fungal colonization in the EBC of 70% of enrolled subjects with asthma was detected (none detected in the controls). An overlap of fungal microbiome in EBC and sputum was observed (100% of overlap). Fungal colonization was higher in subjects without atopic, obesity, and severe and uncontrolled asthma.*

Conclusion: *When considering the high morbidity and mortality of patients with severe asthma in whom we found an important fungal airways colonization, we support the use of the analysis of exhaled fungal microbiome in these subjects.*

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Asthma is a complex and heterogeneous disease that is triggered and worsened by several different factors.[1] Among risk factors, viral and bacterial infections are largely recognized.[2–7] Most studies support the key role of microbiome in asthma pathogenesis, which indicates that it may lead to chronic lower airway inflammation, impaired mucociliary clearance, increased mucus production, and worsening of asthma.[8–10] Several researchers investigated virus and bacteria in airways of subjects with asthma but only a few studied fungi, which is evidence of a gap in knowledge in this important field.[11–17] Confirming the need for further insights on fungi colonization in patients with asthma are epidemiologic studies in the United States and Europe that associated mild sensitivity, particularly to *Alternaria* and *Cladosporium*, with the development, persistence, and severity of asthma.[6,9,10] Other fungi, including *Candida, Penicillium,* and *Curvularia* species have also been involved in asthma.[14] Although fungal sensitization is common in severe asthma, clinical relevance of fungal sensitization and its relationship with airways colonization by fungi remain unclear.[12] In light of the actual interest in this lacking field, the term severe asthma associated with fungal sensitization has been coined to illustrate the high rate of fungal sensitivity in patients with severe asthma and improvement with antifungal treatment.[11] It is important to state that severe asthma is an important phenotype, characterized by a complex management, because it is frequently accompanied by difficult, and often not reached, control.[18] Furthermore, these patients use a significant proportion of medical resource in terms of pharmacologic treatments, hospital admissions or the use of emergency services, and time off school and work.[8,14]

The Global Initiative for Asthma (GINA) guidelines report the possibility to study airways colonization of subjects with asthma with the induced sputum.[1] The procedure is not always effective and is contraindicated in patients with severe asthma because the hypertonic saline solution may cause bronchoconstriction.[19] Lately, the exhaled breath condensate (EBC) has been largely studied as an alternative sample to the induced sputum: it also comes from airways, but its collection is completely noninvasive, which requires one to only breathe into a mouthpiece connected to a

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condenser at tidal volume for 10 minutes.[20] In the EBC, it is possible to analyze either soluble and OMIC markers.[19–22] Recently, our group analyzed fungal colonization of the EBC of patients with lung cancer, which showed a presence of *Aspergillus niger*, or *Aspergillus ochraceus*, or *Penicillium* species, and demonstrated the suitability of the EBC for the study of airways microbiome.[23]

The aim of our study was to analyze, for the first time to our knowledge, fungal microbiome in the EBC of subjects with asthma. We further studied differences in airway fungal colonization between nonatopic and atopic asthma and possible associations between fungal microbiome, anthropometric (sex, age, body mass index), and clinical-functional data (severity of asthma, control, symptoms, exacerbations, cytologic type of inflammation, and comorbidities).

METHODS

Patients

Forty-seven consecutive subjects with asthma (37.7 ± 4.5 years), 28 affected by atopic asthma and 19 affected by nonatopic asthma, and 20 healthy controls (39.2 ± 3.9 years) were recruited for the study from the outpatient facility of the Institute of Respiratory Diseases of the University of Foggia, Italy (Table 1). Written informed consent was obtained from all the subjects, and the institutional ethics committee approved the study. Author contributions included the following: G.E. Carpagnano: substantial contributions to conception and design of the study; M. Malerba: interpretation of data; D. Lacedonia: interpretation of data; A. Susca: acquisition of data; A. Logrieco: revision of manuscript; M. Carone: revision of manuscript; G. Cotugno: acquisition of data; G.A. Palmiotti: acquisition of data; and M.P. Foschino-Barbaro: final approval of the version to be published.

The subjects with asthma were classified and treated according to GINA guidelines.[1] All the subjects with asthma were assessed during a period of stability and at least 4 weeks after an upper respiratory tract infection. During the first visit, a complete baseline questionnaire that requested information about medical history was administered to all the subjects, who were then given a physical examination, atopy assessment, and spirometry with bronchial obstruction reversibility test. During the second visit, the subjects underwent EBC collection followed by the fractional exhaled nitric oxide (FeNO) and the exhaled temperature measurement, and, finally, by sputum induction.

Induced Sputum Collection and Processing

According to the method described by Spanevello *et al.*,[24] sputum was induced in healthy subjects and in subjects with mild asthma, and was analyzed after selection of plug from entair sputum. Five healthy controls and six subjects with asthma were not able to produce adequate sputum samples (defined as containing at least 500 nonsquamous cells), and their expectorates were discharged.

EBC Collection and Processing

In one sitting, from each patient at the time of diagnosis, 1 mL of EBC was collected as previously described. To exclude saliva contamination, the amylase activity was analyzed in the EBC.[25]

Fungal Culture and Identification

The EBC and the sputum were transferred after the collection onto freshly prepared Dichloran Rose-Bengal Chloramphenicol Agar (Thermo Fisher Oxoid, Rodano, Milan, Italy), a selective medium for yeasts and molds. The plates were incubated at 25°C for 7 days and examined daily. Fungal colonies were identified to the genus level based on their morphologic characters by focusing attention on filamentous fungi, which represent, of the genera, the greatest risk for human and animal health: *Aspergillus, Penicillium, Fusarium, Stachybotrys, Alternaria,* and *Cladosporium.*[26] To contribute to the validation of the EBC in the study of the exhaled microbiome, we also performed the analysis in paired induced sputum from each of the enrolled patients.

Measurement of Exhaled NO

A rapid-response chemiluminescence NO analyzer (NIOX MINO; Aerocrine, Cosmed, Roma, Italy) was used to quantify NOs as previously reported.[27,28]

Exhaled Breath Temperature Measurement

Exhaled breath temperature was measured with an X-Halo device (Delmedica Investments, Singapore) according to previously validated methods.[29,30]

Statistical Analysis

To assess the difference between the fungi positivity in the induced sputum and the EBC in the different groups (atopic, nonatopic; obese, nonobese; controlled, uncontrolled; exacerbator, nonexacerbator; symptomatic, asymptomatic; atopy to miceti, atopy to pneumoallergens; comorbidity, no comorbidity; eosinophils, no eosinophils in sputum; neutrophils, no neutrophils in sputum; high FeNO, normal FeNO; and smokers, nonsmokers) we performed a test on the equality of proportions and calculated the Z test when considering a p value of <0.05 as statistically significant.

RESULTS

Anthropometric and clinical data (lung function, airways inflammation, exacerbations, symptoms, asthma

Table 1 **Anthropometric and clinical data of the subjects**

	Atopic Asthma (n = 28)	Nonatopic Asthma (n = 19)	Healthy Controls (n = 20)
Age, mean (SD)	54.1 ± 13.6	55.4 ± 15.3	39.2 ± 3.9
Sex, M/F	25/3	11/8	12/8
Body mass index, kg/m^2	21.1 ± 10.8	19.8 ± 12.2	20.3 ± 8.2
Smoker, yes/no	2/26	1/18	2/18
Pack-years (ex-smokers), mean (SD)	12.1 ± 9.6	24.3 ± 23.1	20.3 ± 6.2
ACTp	20.4 ± 5.1	19 ± 4.6	—
Positive allergic sensitization to mold spore, no.	13	0	0
Exacerbations, no./y			
>2	10	6	—
1	9	9	—
0	9	4	—
Symptoms			
Yes (n)	8	6	—
No (n)	20	13	—
FEV_1%, mean (SD)	82.2 ± 16.7	77.2 ± 16	100.1 ± 3.6
FEV1, L, mean (SD)	2.03 ± 0.64	2 ± 0.58	2.9 ± 0.2
FVC%, mean (SD)	104.6 ± 17.1	96.05 ± 14.7	102.4 ± 2.9
FVC, L, mean (SD)	3.03 ± 0.8	2.9 ± 0.8	3.57 ± 0.8
FEV_1:FVC, mean (SD)	65.5 ± 9.3	64.8 ± 9.8	88.6 ± 3.6
Asthma severity, no.			
Intermittent	10	7	—
Mild	7	2	—
Moderate	5	6	—
Severe	6	4	—
Duration of asthma, no.			
<5 y	14	8	—
<10 y	10	5	—
>10 y	4	6	—
Comorbidity, no.			
Yes	8	16	—
No	20	3	—
FeNO (50), ppb, mean (SD)	21.89 ± 13.9	29.9 ± 20.3	6.2 ± 2.1
FeNO (350), ppb, mean (SD)	8.85 ± 6.3	10.64 ± 6.9	3.1 ± 0.9
X-Halo, °C, mean (SD)	32.26 ± 3.32	30.14 ± 3.12	29.2 ± 2.1
Induced sputum, %, mean (SD)			
Eosinophilia	52.5 ± 4.9	2.10.5	0.6 ± 0.8
Neutrophilia	66.2 ± 12.3	78.25 ± 18.7	27.3 ± 13

ACT = asthma control test; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; FeNO = fractional exhaled nitric oxide.

severity, duration of asthma, comorbidities) of subjects with asthma and with atopic and subjects with asthma and without atopy are reported in Table 1. We were able to find that there was a trend toward higher fungal colonization in the EBC of 70% of enrolled subjects with asthma than in the controls (0%) (Fig. 1 *a*).

Ninety-four percent of the subjects with asthma turned out to have fungi colonized in the EBC by *Cladosporium* species, 21% by *Alternaria* species, and 24% by *Penicillium* species. (Fig. 2). Fungi positivity in the EBC was always confirmed in paired sputum (Fig. 1 *b*). The EBC was found to have identical sensibility compared with the induced sputum for fungi detection (100% of matched positivity). When analyzing subjects with asthma, we found that the fungal colonization was higher in the subjects with asthma and without

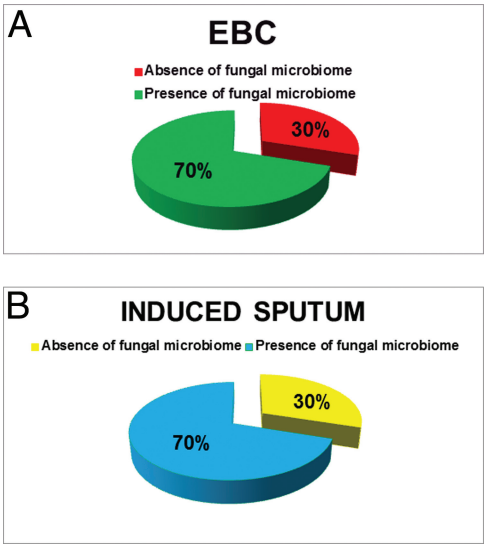

Figure 1. *Fungal microbiome in (a) the exhaled breath condensate (EBC) and (b) in the induced sputum of patients with asthma.*

atopy than in subjects with asthma and with atopy (84% versus 64%) ($p = 0.07$), in subjects who were obese compared with subjects who were not obese (73% versus 68%) ($p = 0.03$), in subjects with more severe asthma (63% in intermittent asthma versus 90% mild persistent asthma versus 100% moderate persistent-to-severe asthma), in uncontrolled than in controlled asthma (73% versus 68%) ($p = 0.03$), in subjects with asthma and with symptoms than in those without symptoms (73% versus 68%) ($p = 0.03$), in subjects with atopy to mycete compared with those with other positivity to pneumoallergens (89% versus 61%) ($p < 0.05$). No association was found among exhaled fungal positivity and smoking habit, comorbidities, inflammatory cells in the induced sputum, exhaled breath temperature, exhaled bronchial and alveolar NO, exacerbations of disease, and duration of asthma.

DISCUSSION

In this study, we tested the EBC for the analysis of fungal microbiome in airways of subjects with asthma. To more effectively verify whether EBC reflects airways infection, we analyzed the fungal microbiome in the EBC and the paired induced sputum, whose use is validated in clinical practice by GINA guidelines, which reports a complete overlap of results.[1] Finally, We observed an increase of fungi in airways of subjects with severe and uncontrolled asthma.

The strength of this study was that, for the first time to our knowledge, a noninvasive sample, the EBC, was used to study the microbiome of subjects with asthma, which compared results in a sample, recognized by the clinical community for the microbiologic analysis in asthma, *i.e.,* the induced sputum. The overlap of microbiome results in EBC and induced sputum led us to support the clinical value of the EBC in the study of fungi in the airways of subjects with asthma that seems to have a direct influence on the severity of the disease. However, when considering that this was a preliminary analysis that investigated an interesting but unexplored field of asthma such as the fungal microbiome, this study presented a limitation in that a molecular biology analysis to better characterize fungi was not carried out.

The main result of our work was to find an elevated presence of fungal colonization in the airways of subjects with asthma, superior to that previously reported by Pashley *et al.*[13] We believe that different results in the incidence of fungi might be due to a different geographic area of provenance of subjects enrolled (United Kingdom versus Italy), surely characterized by a diverse colonization and growth of fungi. As expected by the results of a recent publication from Knutsen *et al.*,[14] we observed that there was an association between exhaled fungal microbiome and asthma, and that this relationship seemed to be influenced by the severity of the disease.[31] We found higher percentages of fungi in the subjects with more severe, uncontrolled asthma and in those patients with symptoms.

Other studies also confirmed this link, as the study by Pashley *et al.*,[13] which describes a positivity in sputum fungal culture in patients with asthma and with reduced lung function, and the study by Knutsen et al.,[14] which reported sensitivity to *Aspergillus fumigatus* associated with severe persistent asthma in adults and Pashley *et al.*,[13] who reported a mycotic positivity of airways of subjects with asthma with persistent symptoms and reduced controls of the disease. We also described an increased incidence of fungi in airways of subjects with asthma and who were obese compared with subjects who were not obese, data that further confirmed the association between colonization and severity when considering that obesity acts as a trigger and worsening factor for asthma.[32]

Other researchers discussed the microbiome of the home, and it is of great interest because of its possible impact on health, and they identified some of the factors that determine the richness, evenness, and diversity of the home's fungal and bacterial microbiomes.[33] Sharpe *et al.*,[34] for example, supposed that indoor dampness increases the risk of indoor fungal growth, which is believed to increase the risk of having asthma, exacerbation of asthma symptoms, or both. They reported the presence of *Cladosporium, Alternaria, Aspergillus,* and *Penicillium* species in higher concentrations in homes of participants with asthma.

Fungal colonization has been studied in association with indices of asthma severity, as the number of exacerbations per year. In this regard, which is different from Zhu *et al.*,[15] we did not observe more infections per year in our subjects who were positive to fungi

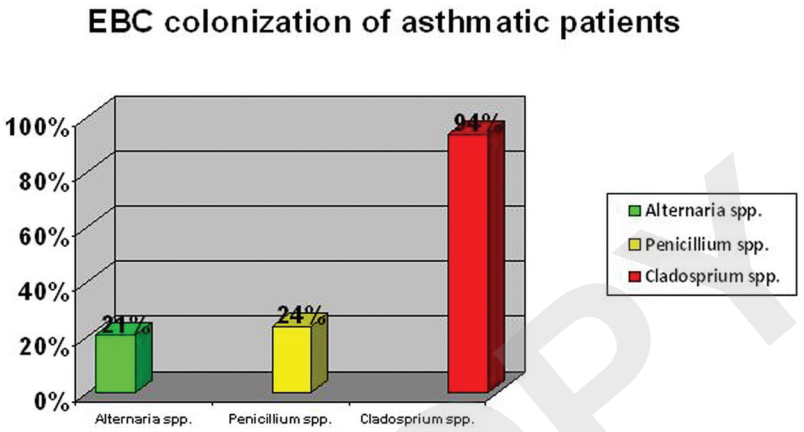

Figure 2. *Fungal colonization type in the exhaled breath condensate (EBC) of patients with asthma.*

colonization. This result did not surprise us in consideration of the role that microorganisms, *e.g.,* fungi, have in the immunomodulation. Analysis of our data indicated that the persistence of these microorganisms in airways might increase bronchial hyperactivity, influencing clinical characteristics of asthma as the severity, the persistence of symptoms, and the control of the disease. We also reported, for the first time, that the fungal colonization was higher in subjects with asthma and no atopy than in subjects with asthma and atopy. These data could simply be due to the worsened clinical condition of patients with nonatopic asthma, but this merits further specific studies regarding this aspect.

In this study, we further observed that subjects with atopy and with positivity to fungi (positive prick test) showed a higher fungal colonization compared with those with positivity to other pneumoallergens. Similarly, Knutsen *et al.*[14] previously reported an association between sensibility to fungi and mold, particularly the *Alternaria alternata* and *Cladosporium herbarum*, with the development, persistence, and severity of asthma.

We were unable to find any correlation among fungi positivity and sex, age, comorbidities, and duration of asthma that would help us in better defining the pathogenetic role of airways fungal colonization. Furthermore, we did not observe any correlation with inflammatory cells in sputum, exhaled bronchial and alveolar NO, and breath temperature. However, the number of patients enrolled was low and justified our results, which we intend to verify on a larger population in consideration of the possible clinical consequences and therapeutic options that they might have.

In this study, we proved that the EBC is a suitable sample for the study of the microbiome. Although other samples are usually indicated for microbiologic analysis of airways of subjects with asthma, as with the induced sputum, the method to obtain the EBC is completely noninvasive and safe, and becomes very important when subjects to be analyzed are those with asthma and especially those with severe and uncontrolled disease. Furthermore, the use of the induced sputum, proposed by GINA guidelines,[1] for the microbial analysis of airways is not proposable in subjects with severe asthma in which the inhalation of hypertonic saline solution, foreseen for the induction, is contraindicated. Because we found fungal positivity particularly in subjects with severe and uncontrolled asthma, the use of the EBC could be an interesting clinical alternative for the microbiologic analysis of airways. Notwithstanding the interest that our results open in the field of noninvasive study of airways of subjects with asthma, we have to underline that, at the moment, the EBC is a method whose collection has not been standardized and in which the study of microbiome must to be validated before thinking in terms of clinical diagnosis. The complete overlap of fungal positivity in the EBC and induced sputum (100%) enabled us to support the use of the EBC instead of the induced sputum in microbiologic study of airways at least in subjects unable to undergo this method.

In consideration of the unanswered question regarding the molecular characterization of fungi[35] because it was a preliminary study, we are planning a larger future study in which we will perform a morphologic and genetic characterization of microbiome of patients with asthma.

CONCLUSION

We demonstrated, for the first time to our knowledge, the possibility to study the fungal microbiome in the EBC of subjects with asthma and showed that the fungal colonization of airways is associated with more severe asthma, with an increased persistence of symptoms, and with less control of the disease. Although we just observed a trend toward the increase, which requires a larger number to potentially become significant, in light of our results, we believe the study and the characterization of the fungal microbiome in airways of subjects with asthma, particularly those with severe asthma, to be useful because it might give an

important contribution to the comprehension of pathogenetic mechanisms of asthma, some of which are still unknown.

Nowadays, the study of the phenotype of severe uncontrolled asthma is very important because it is the main form that does not respond to treatment, which causes an increase of morbidity and mortality for asthma and is associated with high direct and indirect health costs.

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