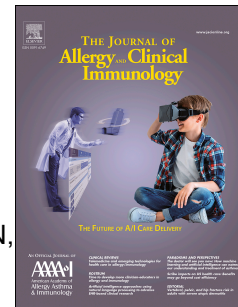


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Type 2-high asthma is associated with a specific indoor mycobiome and microbiome

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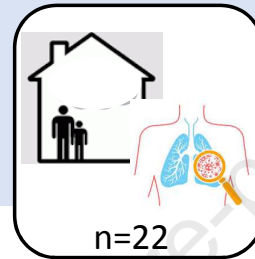
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Patients with type 2-high severe asthma revealed a specific indoor microbial environment

Microbiome and Mycobiome analysis of induced sputum samples (Respiratory samples (R)) and electrostatic dust collector located at patient's bedroom (Indoor samples (I))

The analysis were based on bacterial (V3-V4 locus of 16S gene) and fungal (ITS2 region of rDNA) amplification using a 250-bp paired-end technology on MiSeq (Illumina) platform.



Severe asthma

Main findings

(1) FeNO level is correlated with indoor fungal Chao1 indexes

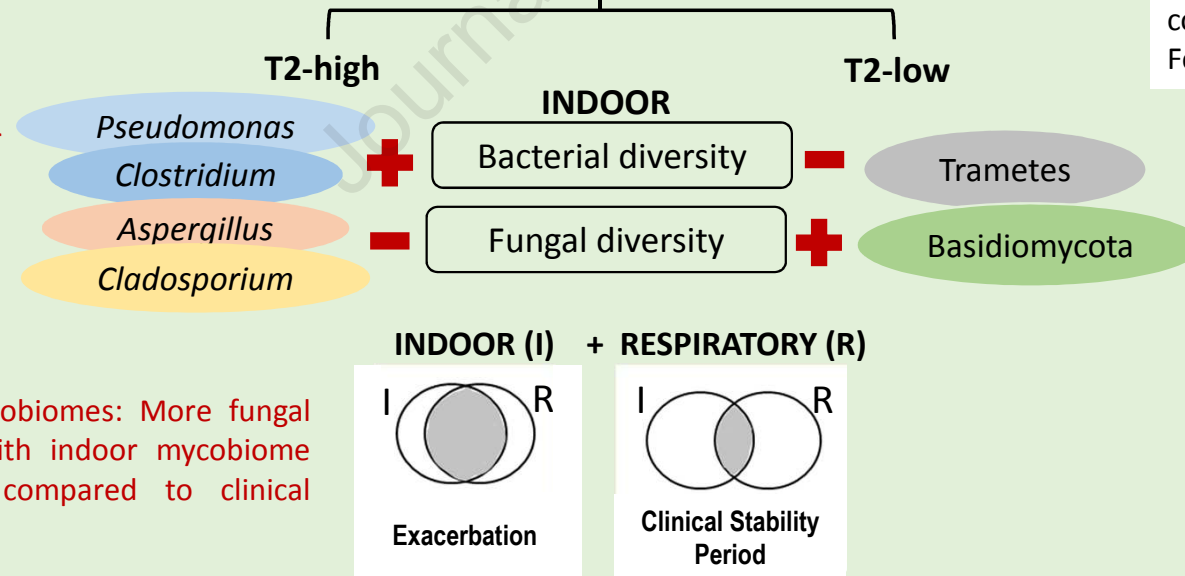
(2) Beta-diversity of indoor fungal communities is clustered according to T2 endotypes

(3) Indoor and Respiratory mycobiomes: More fungal taxa are significantly shared with indoor mycobiome during exacerbation periods, compared to clinical stability periods

Abbreviations:

Chao1 index: Alpha-diversity index estimating the number of species in a community

FeNO: Fraction of exhaled nitric oxide



Type 2-high asthma is associated with a specific indoor mycobiome and microbiome

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DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST. LEV, CU and SF are employees of GenoScreen. POG reports personal fees and non-financial support from AstraZeneca, Boehringer Ingelheim, Chiesi, GlaxoSmithKline, Novartis and Sanofi outside the submitted work. PB is the medical coordinator of COBRA, which received grants from AstraZeneca, GlaxoSmithKline, Roche, Chiesi, Novartis and Legs Poix foundation. Moreover, PB reports grants and personal fees from Novartis; personal fees and non-financial support from Chiesi; grants, personal fees and non-financial support from Boehringer Ingelheim; personal fees and non-financial support from AstraZeneca; personal fees and non-financial support from Sanofi; personal fees from Menarini; and personal fees from TEVA outside the submitted work. In addition, PB has 3 patents (i) (EP N°15152886.6, *i.e.*, New compositions and methods of treating and/or preventing Chronic Obstructive Pulmonary Disease), pending; (ii) (EP N°3050574, *i.e.*, Use of plerixafor for treating and/or preventing acute exacerbations of chronic obstructive pulmonary disease), delivered; (iii) (EP N°20173595.8, *i.e.*, New compositions and methods of treating COVID-19 Disease), pending. LD was invited to the ERS international congress 2019 as speaker (PG 41000/45500-14200). All other authors report no relevant conflicts of interest.

ABSTRACT.

Background: The links between microbial environmental exposures and asthma are well documented, but no study has combined deep-sequencing results from pulmonary and indoor microbiomes of asthmatic patients with spirometry, clinical and endotype parameters.

Objective: The goal of this study was to investigate the links between indoor microbial exposures and pulmonary microbial communities and to document the role of microbial exposures on inflammatory and clinical outcomes of patients with severe asthma (SA).

Methods: Fifty-five SA patients from the national COBRA cohort were enrolled for analyzing their indoor microbial flora through the use of electrostatic dust collectors (EDCs). Among these patients, 22 were able to produce sputa during stable or pulmonary exacerbation periods and had complete pairs of EDC and sputum samples, both collected and analysed. We used amplicon targeted metagenomics to compare microbial communities from EDC and sputum samples of patients according to type 2 (T2)-asthma endotypes.

Results: Compared to patients with T2-low SA, patients with T2-high SA exhibited an increase in bacterial alpha-diversity and a decrease in fungal alpha-diversity of their indoor microbial floras, the latter being significantly correlated with FeNO levels. The beta-diversity of the EDC mycobiome significantly clustered according to T2 endotypes. Moreover, the proportion of fungal taxa in common between sputum and EDC samples was significantly higher when patients exhibited acute exacerbation.

Conclusion: These results illustrated, for the first time, a potential association between the indoor mycobiome and clinical features of SA patients, which should renew interest in deciphering the interactions between indoor environment, fungi, and host in asthma.

KEY MESSAGES.

- The indoor mycobiomes and microbiomes of severe asthma (SA) patients harbour a distinct signature according to T2 endotypes, with lower fungal diversity but higher bacterial diversity associated with T2-high SA.
- The indoor environments of patients with T2-high have significant enrichment of medically relevant fungi and bacteria, such as *Aspergillus*, *Candida*, *Sphingomonas* and *Pseudomonas*.
- The respiratory mycobiome of SA patients shares more fungal taxa with the indoor mycobiome during exacerbation than during stable periods.

74

75 **CAPSULE SUMMARY.**

76 This study is the first to date that investigates both the endogenous and the exogenous
77 mycobiomes and microbiomes of asthmatic patients and reveals associations between indoor
78 microbial communities, exacerbation, and T2 of severe asthma.

79

80 **KEYWORDS.** Indoor environment; mycobiome; microbiome; severe asthma; FeNO; Type 2
81 asthma

82

83 **ABBREVIATIONS USED.**

84 BMI: Body mass index

85 BAL: Bronchoalveolar lavage

86 COBRA: Cohort of Bronchial obstruction and Asthma

87 EDC: Electrostatic Dust Collector

88 ERS: European Respiratory Society

89 EXA: Exacerbation of asthma

90 FeNO: Fraction of exhaled nitric oxide

91 ICS: Inhaled corticosteroid

92 IL: Interleukin

93 ITS2: Internal transcribed spacer 2

94 LABA: Long acting beta2 agonist

95 LDA: Linear discriminant analysis

96 LAMA: Long acting muscarinic antagonist

97 NGS: Next generation sequencing

98 OCS: Oral corticosteroid

- 99 OTU: Operational taxonomic unit
- 100 PCoA: principal coordinates analysis
- 101 PERMANOVA: Permutational multivariate analysis of variance
- 102 qPCR: Quantitative polymerase chain reaction
- 103 SA: Severe asthma
- 104 STB: Stable state
- 105 T2: Type 2
- 106 VRC: Voriconazole
- 107 V3-V4: Variable 3-variable 4 of the 16S rRNA encoding gene

INTRODUCTION

Asthma is a complex, chronic inflammatory disease of the airways that affects 339 million people worldwide (1,2). Despite recent advances in new treatments and notable efforts to elucidate asthma pathophysiology (2), patients with severe asthma (SA) remain at high risk for complications, exacerbations, poor quality of life and increased mortality and morbidity (1,2). According to the definition of SA proposed by the international ERS/ATS Task Force (3), SA is still recognized as a major unmet need, with an overall prevalence estimated at 5-10% (1–3) and accounting for approximately 50% of asthma-associated health care costs (1). Similar to non-severe asthma, SA is currently accepted as a heterogeneous disease comprising multiple endotypes that combine clinical characteristics with several identifiable mechanistic pathways (3). Developments in biologic medications to treat asthma have allowed for specific endotypes to be targeted, such as allergic asthma, eosinophilic asthma and, more recently, type 2 (T2)-high asthma. It is now standard practice to differentiate SA patients in T2-high and T2-low asthma based on the levels of both the fraction of exhaled nitric oxide (FeNO) and the blood eosinophil counts (4). SA is also associated with microbial lung dysbiosis, which activates the inflammasome and other induced pathways (5–7). In agreement with the hygiene hypothesis, recent findings have demonstrated that exposure to environmental microbes significantly decreases the incidence of wheezing illnesses in young children with a genetic susceptibility at chromosome 17q21 (8). Moreover, the ubiquitous distribution of fungal spores leads to their regular inhalation as an inevitable consequence of breathing (9,10). Exposure to environmental microbes, especially the fungal indoor environment, is associated with a wide range of adverse health effects, including asthma, and can influence asthma severity (8,11–15). In the present pilot study, our goal was to evaluate the relationships between bacteria and fungi in sputum samples and from dust particles from patients with T2-high and T2-low SA.

133 To this end, we examined whether the indoor fungal and bacterial flora (exogenous
134 mycobiome and microbiome, respectively) are associated with clinical parameters in SA
135 patients and how the exogenous and endogenous mycobiomes and/or microbiomes were
136 connected.
137

METHODS

Patients

Fifty-five patients from the national COhort of BRonchial obstruction and Asthma (COBRA, Ethics committee number: 2008-A00294-51/1) (16) suffering from SA based to the ERS/ATS guidelines and followed at the University Hospital of Bordeaux were included in the COBRA-ENV study. The COBRA-ENV protocol (Number: 2017/68) was approved by the ethics committee: “CPP du Sud-Ouest et Outre Mer III”.

Demographic features, clinical characteristics, lung function and induced sputum test results were determined at COBRA visits as previously described (16). Patients with T2-high SA were defined according to either a level of FeNO higher than 25 ppb or a blood eosinophil count higher than 300 cell/mm³ (7). In contrast, patients with T2-low SA were defined according to both a FeNO level lower than 25 ppb and a blood eosinophil count lower than 300 cell/mm³.

Sputum and indoor dust samples

Induced sputa were successfully collected from 22 patients, as previously described (17). Indoor dust samples were collected using an electrostatic dust collector (EDC). Briefly, each EDC consists of a 20 cm × 17 cm textile surface that is used to catch fungi and bacteria during the exposure period (18). This textile device is mounted in a plastic folder that is left open for 10 weeks in a horizontal position with the textile exposed to the room air such that the settling dust is passively collected (18,19). Using the same protocol, EDCs were placed in patient bedrooms, opened on the same date (*i.e.*, December 21st, 2017) and exposed for the same duration (*i.e.*, 10 weeks), as described previously (19).

DNA extraction, library preparation, deep-sequencing, and taxonomic assignment

DNA was extracted from EDC and sputum samples using a DNeasy PowerSoil kit (Qiagen, France). Negative extraction controls (250 µl of DNA-free water, as an extraction blank) were processed using the same protocol. The microbial diversity and taxonomic composition of samples were assessed using the V3-V4 region of the bacterial 16S rRNA gene and the internal transcribed spacer 2 (ITS2) region of the fungal rDNA following optimized and standardized library preparation protocols from Metabiote® (Genoscreen, Lille, France) (20). The primers used to amplify the V3-V4 and ITS2 loci were 16S-Forward (TACGGRAGGCAGCAG) and 16S-Reverse (CTACCNGGGTATCTAAT) as well as ITS2-Forward (GTGARTCATCGAATCTTT) and ITS2-Reverse (GATATGCTTAAGTTCAGCGGGT), respectively. In addition to the extraction blanks, two negatives controls (one library blank and one unexposed EDC but analysed following the same process as the exposed EDCs) and two positive controls (artificial bacterial and fungal communities) were used to valid the experimental procedures. Briefly, PCR amplification was performed using barcoded primers (at 0.2 µM final concentration), with an annealing temperature of 50°C for 30 cycles. PCR products were purified using magnetic beads, quantified according to the protocol provided by GenoScreen, and mixed in equimolar amounts. NGS sequencing was performed using 250-bp paired-end technology on a MiSeq platform (Illumina, San Diego, CA, USA) at GenoScreen (GenoScreen, Lille, France).

The resulting raw sequences were subjected to a cleaning process as follows: i) sorting the sequences according to the indexes and the 16S and ITS2 primers using CutAdapt, with no mismatch allowed within the primers sequences; ii) quality-filtering using the PRINSEQ-lite PERL script by truncating bases at the 3' end with Phred quality scores <30; and (iii) generating a paired-end read assembly using FLASH with a minimum overlap of 30 bases and a >97% identity. The bioinformatic analysis was performed on a fully automated (Metabiote® OnLine v2.0) pipeline using QIIME v 1.9.1 software (21). Following pre-processing steps, the

full-length 16S and ITS2 amplicons were assessed for chimeric sequences using an in-house method based on Usearch 6.1. Then, a clustering step was performed to group similar sequences with a defined nucleic identity threshold (97% identity for an affiliation at least at the genus level for the V3-V4 locus and at the species level for the ITS2 locus) using Uclust v1.2.22q (22) through an open-reference OTU picking process and a complete-linkage method to generate groups of sequences or “operational taxonomic units” (OTUs). An OTU cleaning step was performed to eliminate singletons. For each OTU, the most abundant sequence was used as a reference sequence and was taxonomically compared to reference databases, including the Greengenes database (release 13_8) for bacterial OTUs and the Unite database (release 7.2) for fungal OTUs using the RDP classifier method v2.2 (23). A final table with all of the identified OTUs was generated comprising the number of sequences for each sample related to each OTU and their respective taxonomic affiliation. Alpha-diversity metrics within samples (Chao1, Simpson and Shannon indexes) were generated from this OTU table using QIIME v1. The sequence information has been deposited in the NCBI Sequence Read Archive under the accession number PRJNA635002.

Estimation of the EDC fungal load using a qPCR assay

The fungal load for each EDC was estimated with a universal qPCR assay that quantifies rDNA (in pg per EDC). Briefly, previously published primers and probe (24), Taqman® Gene Expression Master Mix (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) and a 7900HT Sequence Detection System were used to perform qPCR, and the protocol is detailed in the online supplementary material.

Data analysis

The R programming environment and packages were used for statistical analyses. Non-parametric tests (Man-Whitney test for quantitative variables) and Fisher's exact test (for qualitative variables) were used when appropriate. Linear relationships between FeNO levels and Chao1 fungal diversity was estimated using Pearson's correlation coefficient. Differences were considered statistically significant at p-values below 0.05.

Alpha- and beta-diversities were determined to examine changes in microbial community structures between EDCs and sputa, according to the clinical data. The principal coordinates analysis (PCoA, after a Bray-Curtis distance analysis) method was used to visualize overall microbial differences among groups. The microbial core was defined using the Venn Diagram function in the RAM package.

To compare alpha-diversity estimates across the clinical parameter of patients, Mann-Whitney tests were performed, while Wilcoxon tests were used when pairs of sputum and EDC were compared. After a cumulative sum scaling normalization, sample clustering hypotheses were tested using PERMANOVA analysis, with a dispersion among groups assessed using the Betadisper function to complete the beta-diversity analysis. Finally, differentially abundant OTUs between groups were identified using linear discriminant analysis (LDA) effect size (LEfSe), as previously described (25,26). According to Segata et al (25), we applied LEfSe with the default parameters (alpha value for Wilcoxon tests was set at 0.05, logarithmic LDA scores threshold set at 2.0, and bootstrapping over 30 cycles) to identify indoor taxonomic biomarkers that characterize the differences between patients with normal and elevated FeNO level using T2 endotypes as covariables.

Additional details of patient characteristics and sampling, and methodological procedures are provided in the online supplementary material.

RESULTS

Study population

Fifty-five SA patients were enrolled, but only 22 SA patients had complete pairs of EDC and sputum collected and analysed (Supplemental Figure E1). Patient characteristics from the T2-high and T2-low groups are summarized in Table 1. Except for FeNO levels, the characteristics of patients with T2-high SA were not significantly different from those with T2-low SA. Similarly, sputum microbial cultures and indoor fungal loads measured by qPCR were not significantly different between the two SA groups (Table 1).

Indoor mycobiomes and microbiomes significantly differed according to T2 endotypes

Patients with T2-high SA showed an increase in the three alpha diversity metrics for the EDC bacterial communities compared to those observed for the T2-low SA patients, but only the Shannon and Simpson indexes were statistically significant (Fig 1, A). In contrast, the same three alpha diversity metrics for the EDC fungal communities were lower for the patients with T2-high SA compared to those observed for the T2-low SA patients, but only the Shannon indexes were statistically significant (Fig 1, B). FeNO levels were significantly correlated with the alpha-diversity of the mycobiome as measured by Chao1 index values (Fig 1, C). The beta-diversity of the EDC fungal communities significantly clustered according to the T2 endotypes (Fig 1, D).

Indoor mycobiomes and microbiomes of patients with T2-high SA were significantly enrichment for medically relevant fungi and bacteria

The bacterial composition of the indoor microbiome was dominated at the phylum level by Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, with the most abundant OTUs annotated as *Paracoccus* spp., *Acinetobacter* spp. and *Streptococcus* spp. (see Fig E2). The

261 fungal community was represented by the two major phyla, Ascomycota and Basidiomycota,
 262 with the most abundant OTUs annotated as *Epicoccum nigrum*, *Cladosporium*
 263 *cladosporioides*, *Cladosporium sphaerospermum*, *Malassezia restricta* and *Trametes*
 264 *versicolor* (see Fig E3). All bacterial and fungal OTUs were then submitted to the LEfSe
 265 method using T2 endotypes as covariables.

266 LEfSe analysis results indicated significant community enrichment of EDCs from patients
 267 with FeNO >25 ppb and T2-high SA as a covariable, which was composed of 7 families and
 268 10 genera of bacterial OTUs (Fig 2, A) and 2 genera plus 5 species of fungal OTUs (Fig 2, B).
 269 While enrichments of EDCs from patients with low FeNO levels were primarily composed of
 270 environmental Basidiomycota OTUs (Fig 2, B), those from patients with FeNO >25 ppb and
 271 T2-high SA as a covariable were composed of fungal and bacterial OTUs known to be
 272 medically relevant in allergies or asthma (i.e., *Cladosporium*, *Aspergillus*, *Epicoccum*,
 273 *Candida*, *Rhodotorula*, *Pseudomonas*, *Pedobacter*, *Erwinia*, *Sphingomonas*,
 274 *Sphingomonadaceae*, or *Methylocystaceae*) (1,9,10).

275 276 **Indoor and sputum samples of patients with asthma exacerbation share a mycobiome** 277 **core composed of medically relevant fungi**

278 Twenty-two patients were able to produce induced sputum during clinically “stable” (n=10,
 279 including 9 T2-high SA and 1 T2-low SA patients) or “exacerbated” (n=12, including 7 T2-
 280 high SA and 5 T2-low SA patients) periods defined at COBRA visit. Both the bacterial and
 281 fungal diversities of these sputa were lower than those of the corresponding EDCs (Fig 3, A).
 282 The taxonomic composition of these two types of samples was significantly different (Fig 3,
 283 B-D; see Fig E2-E5). These results revealed the highly individual signature of sputum
 284 mycobiomes and microbiomes compared to that observed for EDCs, which exhibited a very
 285 closely spaced clustering of samples (Fig 3, C and D). For instance, the proportion of

Basidiomycota, a phylum primarily composed of environmental fungi, compared to Ascomycota, which includes medically relevant fungi, was higher in EDCs compared to sputa (Fig 3, B), highlighting the complex microbial ecology of the human respiratory tract.

We did not observe any differences in the bacterial and fungal diversities of these sputum samples according to T2 endotypes (data not shown). In contrast, the proportion of common bacterial taxa between the EDCs and sputa was similar when sputa were collected during exacerbation or at a stable state (Fig 3, E), whereas the proportion of common fungal taxa between EDCs and sputa was significantly higher when sputa were collected during exacerbation period compared to the stable period (Fig 3, F). The Venn diagram results confirmed a limited bacterial core composed of 5 OTUs (Fig 4, A) and a large fungal core composed of 27 OTUs (Fig 4, B).

We identified the core microbes between pairs of sputa and EDCs according to the absence or presence of an asthma exacerbation and confirmed that only one OTU was shared between the bacterial core and the clinical states (i.e., *Sphingomonas* genus, Fig 4, A). Surprisingly, 9 fungal OTUs (i.e., *Hyphodontia radula*, *Resinicium bicolor*, *Phlebia radiata*, *Penicillium brevicombactum*, *Vishniacozyma victoriae* (syn. *Cryptococcus victoriae*), *Sistotremastrum* sp., *Cryptococcus magnus*, *Bjerkandera* sp., and *Botryotinia fuckeliana*, Fig 4, B) were identified as the fungal core and were specifically shared between EDCs and sputa collected during exacerbation. In addition, 17 other OTUs were identified as members of the fungal core independent of the clinical status, which included medically relevant yeasts (i.e., *Saccharomyces cerevisiae*, *Debaryomyces hansenii*, and *Malassezia* species) and moulds (*Aspergillus* and *Cladosporium* species) or environmental Basidiomycota and Ascomycota. Only one *Alternaria brassicae* OTU was shared with sputa collected at a stable state (Fig 4, B). Among these OTUs, several were both microbial core members and significantly enriched

310 OTUs in EDC microbial communities, including *C. sphaerospermum*, *T. versicolor*, and
311 Sphingomonadaceae (Fig 2, B and Fig 4, B).

DISCUSSION

To the best of our knowledge, this pilot study is the first to report that the indoor mycobiomes and microbiomes of SA patients exhibit distinct signatures according to T2 endotypes. Based on pairs of EDC and sputum samples, we compared exogenous and endogenous microbial communities and identified a fungal core comprising medically relevant fungi that was significantly more pronounced when sputa were collected during asthma exacerbations.

Regarding the indoor environment, we clearly demonstrated that a higher bacterial alpha-diversity together with a lower fungal alpha-diversity was associated with T2-high SA patients and that the fungal beta-diversity of EDC communities was clustered according to T2 endotypes. Moreover, the indoor mycobiomes and microbiomes of patients with FeNO >25 ppb and T2-high SA were significantly enriched for medically relevant fungi and bacteria. Among them, we identified changes in the relative abundances of Sphingomonadaceae, Methylocystaceae, *Erwinia*, *Sphingomonas*, *Pseudomonas*, and *Candida*, which is in agreement with previously published data on the gut microbiota and allergy/asthma (27).

Although not exclusively focused on T2 endotypes, several studies have previously investigated the respiratory microbiomes or mycobiomes of patients with varying degrees of asthma severity (28–42). Among these studies, an association between Firmicutes and Actinobacter with SA has been reported (33,35). In addition, an excess of Proteobacteria was shown to be associated with moderately severe to severe asthmatics (33,35), while an excess of *Streptococcus* was shown to be associated with eosinophilic asthma (42). However, little is known regarding the lung mycobiome in chronic respiratory diseases. *Malassezia* yeasts have been shown to be significantly associated with asthma and more recently with exacerbation in cystic fibrosis (26,29). The mycobiomes of SA patients were shown to harbour higher loads of fungi compared to non-severe asthmatic or healthy individuals (28), with a lower diversity observed in T2-high asthma patients (7). A low indoor fungal diversity has been proposed to

be a causal factor in the development of childhood asthma (43), and although a few studies considered adult patients with asthma with respect to its severity and control, none of them referred to T2 endotypes and combined exogenous to endogenous mycobiome and microbiome analyses.

The first studies of indoor microbial ecology used culture-based techniques (44), but have more recently been performed using NGS-based approaches. Very few studies have performed NGS analyses of the indoor mycobiome and/or microbiome in asthma (14,15,37–39, 43,45). Overall, sampling the indoor microbiome by a passive collector such as EDC allows for the recovery of house dust particles, which are a complex mixture of inorganic and organic substances. The indoor mycobiome appears to be a large fungal community assemblage (10,45–51) that is primarily derived from the outdoor environment (38) but also depends on the inhabitants' mycobiomes under selective pressures (10,46). As previously reported (46,48,49,51), we sampled patient bedrooms, which are known to accurately estimate the level of indoor fungal pollution (44). Our NGS results for the EDC mycobiome and microbiome were congruent with these previous studies. Briefly, we identified Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria as the most abundant bacterial phyla in agreement with recently published data (52,53). Within these phyla, *Pseudomonas* and *Sphingomonas* were significantly enriched in the EDCs of patients with T2-high SA. Regarding the mycobiome, *Aspergillus versicolor* and *C. sphaerospermum* have been identified as indoor species correlated to asthma (44,45,52). Using NGS, Dannemiller et al identified numerous fungi that were significantly associated with SA (45). Our results confirmed that several *Cryptococcus* species and *E. nigrum* were associated with SA and that the genus *Epicoccum* was significantly enriched in the indoor mycobiome of T2-high SA patients. Moreover, our indoor data are congruent with the recent identification of

361 *Cladosporium* and *Aspergillus* as dominant enriched genera of respiratory samples from
362 patients with T2-high asthma (7).

363 In addition to viral infections, fungal sensitization and fungal exposure have been shown to be
364 highly associated with asthma exacerbations and was recently reviewed (46). We confirmed
365 that fungal exposure may be a key player during exacerbation, as the fungal OTU proportion
366 shared by EDCs and sputa was significantly higher when sputa were collected during asthma
367 exacerbation. These results support the idea that microbial analysis should not be restricted to
368 a specific type of microorganism (viruses, bacteria, or fungi) but rather should involve
369 analyses of inter-kingdom interactions that may be involved in promoting exacerbations
370 (7,26).

371 FeNO is an endogenous gaseous molecule incorporated into the clinical management of
372 chronic respiratory diseases and is currently recognized as a biomarker of T2 airway
373 inflammation (54). Using conventional mycological methods (*i.e.*, cultures and PCR), FeNO
374 levels of patients with asthma have been related to environmental exposure (55), while the
375 indoor isolation of *A. versicolor* and *Cladosporium* have been associated with higher FeNO
376 levels (56,57), which is in agreement with our results. FeNO levels were shown to be
377 positively correlated with T2 cytokine levels in asthmatics and with serum IL-17A levels in
378 SA patients (58). As adaptive antifungal immunity includes both Th2 and Th17-type CD4⁺ T
379 cells, we hypothesize that a number of the fungi isolated from the indoor mycobiome of SA
380 patients (such as *Aspergillus* and *Cladosporium* species) should contribute to a detrimental
381 immunopathology in SA patients. Once fungi are inhaled and colonize the respiratory airways
382 of patients, they participate in complex microbial-host interactions by producing secondary
383 metabolites that are well-known to be involved in this immune response, such as cell-wall
384 components (β -glucan and/or chitin) or secreted enzymes (proteases and/or glycosidases, or
385 toxins) (59).

While our work is novel, it does have several limitations, including its cross-sectional design and the limited number of fully analysed pairs of EDC plus sputum, which reflects sampling difficulties of the respiratory tract. Indeed, the small number of patients limits the generalizability of our results, especially regarding LEfSe results. For instance, the absence of difference between the T2-low and T2-high SA groups with respect to the exacerbation rate is probably underpowered. We used induced sputa to analyse the endogenous microbiomes and mycobiomes, a sampling method recently proposed as an acceptable and less invasive alternative compared to bronchoalveolar lavage (BAL) samples but with several biases (60-62). In addition, since only patients with higher than 50% predicted post-bronchodilator FEV₁ were allowed to achieve induced sputum, which can be considered to be a bias of selection. As most of the published studies on this topic have focused on asthma-associated microbiomes and mycobiomes, this study suffers from small size limitations and a lack of longitudinal data. Furthermore, amplicon deep-sequencing represents a promising method, but protocols need to be improved and standardized. Indeed, there are many potential biases, ranging from the primer, amplification protocol and NGS machine used to the pipeline and databases selected for analysis (10,63). Thus, further studies are warranted to confidently determine the relationships between the indoor environment, the lung mycobiome and microbiome, the inflammatory response, and the development and severity of asthma.

In summary, in the present study, we identified a correlation between the indoor mycobiome and FeNO levels of patients with SA (Fig 1C), which should renew interest in deciphering the interactions between the indoor environment, fungi, and the host to facilitate the development of novel therapeutic managements (14). Given the role of fungi as potent inducer of airway inflammation (59), an increase in FeNO may serve as an indicator of respiratory inflammation related to exposure to specific moulds that the indoor mycobiome analysis will characterize.

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FIGURE LEGENDS

FIG1. Alpha- and beta-diversities of the EDCs from SA patients according to T2 endotypes(n=22). Alpha-diversity of bacterial (A) or fungal (B) communities from EDCs is shown using Chao1, Shannon and Simpson indexes. Correlation between FeNO levels and Chao1 fungal diversities is presented (C). Beta-diversity of fungal communities (D) is shown. Patients were split in T2-high (dark grey, circles) and T2-low (light grey, triangles) SA.

FIG2. Taxonomic differences between FeNO groups using T2 endotypes as covariables across the 22 EDCs show significant enrichment of specific taxa at the OTU level. Linear discriminant analysis (LDA) and effect size (LEfSe) identified bacterial (A) and fungal (B) taxa differentially enriched according to an LDA score cutoff at 2. The colours (dark and grey) represent each FeNO group (level cutoff at 25 ppb), and the length represents the LDA score, which is the degree of significant difference (in log scale) for a given taxon. As the order of the numerator and denominator when calculating the effect size is determined by alphabetical order, it is possible to obtain an interpretation of the scale of the difference between 2 groups using the absolute values of the effect size, even though the LDA score is negative for a given taxon. Thus, any taxa coloured according to the FeNO variable may be interpreted as taxa being significantly increased in abundance compared to the other FeNO group.

FIG 3. Comparison of diversity and composition of both sputum and EDC samples. The bacterial (left), and fungal diversities (right) based on Shannon indexes are presented for sputum and EDC samples (A). The proportion of Basidiomycota (a phylum primarily composed of environmental fungi) was more abundant in EDCs than in sputa (B). PCoA based on Bray-Curtis dissimilarity between sputum (dark grey, squares) and EDC (light grey,

diamonds) samples for bacterial (C) and fungal (D) communities showed the highly individual signature of sputa compared to EDCs. Proportion of common bacterial (E) or fungal (F) taxa between EDCs and sputa collected at a stable state (STB) or during an exacerbation (EXA). For each OTU, the proportion was expressed as a percentage of the number of reads (*i.e.*, relative abundance).

FIG 4. Microbial core shared between the 22 pairs of indoor and respiratory samples. Venn diagrams show the proportion of common bacterial (A) and fungal (B) taxa (at the OTU level) between EDCs (green section) and sputa collected either during an EXA (sputa collected during an exacerbation, red section) or an STB period (sputa collected during a stable state, blue section). Only OTUs present at least in 20% of each group of sputa (clustered according the presence or absence of an EXA when sputa were collected) and at least in 90% of EDCs were taken into account and represented in the Venn diagrams. The bacterial core (A) showed only one OTU corresponding to *Sphingomonas sp.* shared between EDCs and sputa collected during an exacerbation, and four OTUs belonging to *Streptococcus*, *Paracoccus* and *Haemophilus* species were shared independently from the clinical state of the patients. The fungal core (B) was larger than the bacterial core and was composed of nine OTUs associated with sputa collected during an exacerbation (EXA), one (*Alternaria brassicae*) associated with sputa collected at stable state (STB), and seventeen OTUs (including several medically relevant fungi, such as *Malassezia*, *Aspergillus*, and *Cladosporium* species) belonging to the fungal core independent from the clinical state.

641 **TABLE 1.** Clinical and biological characteristics of patients with SA and complete NGS analysis

Variables	Overall (n = 22)	Type 2-low SA (n = 6)	Type 2-high SA (n = 16)	p- values
Demographic features				
Age (yr)	59.09 [16.84]	52.33 [19.69]	61.62 [15.58]	0.259*
Sex (Males (%))	10 (45.5)	2 (33.3)	8 (50.0)	0.827
BMI (kg/m ²)	26.96 [4.73]	27.31 [2.98]	26.83 [5.32]	0.838
Respiratory data				
FEV1 (% pred)	75.36 [27.60]	79.32 [33.83]	73.77 [25.89]	0.689
FVC (% pred)	91.20 [21.99]	89.18 [28.75]	92.01 [19.83]	0.798
FEV1/FVC (% pred)	68.25 [17.04]	74.84 [15.82]	65.62 [17.31]	0.274
FEF 25-75 (% pred)	49.24 [31.30]	61.83 [36.35]	44.21 [28.85]	0.254
FeNO (ppb)	43.32 [32.74]	15.86 [6.26]	53.62 [32.75]	0.012
Clinical features at COBRA visit				
Very poorly controlled asthma**	13	4	9	0.788
SAFS	10	3	7	1.000
ABPA	3	0	3	0.657
Asthma medication used during the last 6 months**				
ICS	22	6	16	NA****
LABA	22	6	16	NA
LAMA	16	6	10	0.222
Continuous OCS	9	3	6	0.965
Biotherapy	9	3	6	0.965
Gastroesophageal reflux treatment	8	3	5	0.752
Azole treatment	1	0	1 (VRC [†])	1.000
Number of exacerbations during COBRA-ENV period	6.45 [5.29]	9.67 [5.32]	5.25 [4.91]	0.081

Number of OCS courses during COBRA-ENV period	5.45 [5.09]	8.50 [4.89]	4.31 [4.81]	0.085
Number of ATB courses during COBRA-ENV period	4.68 [4.87]	6.83 [3.87]	3.88 [5.07]	0.213

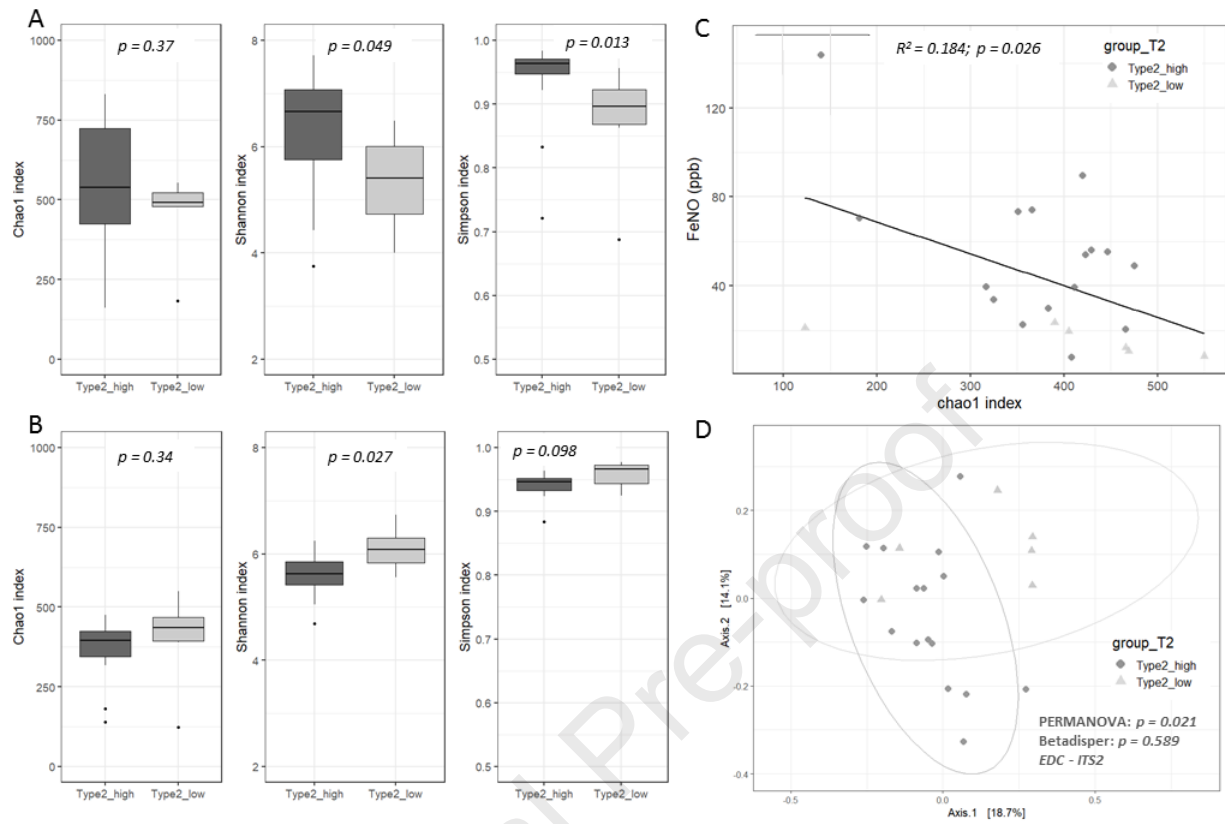
Biological data at COBRA visit

Blood eosinophils count (/mm ³)	526.82 [1184.46]	120.00 [90.77]	679.38 [1367.61]	0.336
Total serum IgE (kUI/L)	308.23 [264.80]	236.67 [268.59]	335.06 [267.03]	0.451
Sputum microbial cultures	19	6	13	0.657
<i>Pseudomonas aeruginosa</i>	1	1	0	0.601
<i>Escherichia coli</i>	1	1	0	0.601
<i>Haemophilus influenzae, parainfluenzae</i>	2	1	1	1.000
<i>Non pneumoniae Streptococcus</i>	2	1	1	1.000
<i>Candida albicans</i>	14	6	8	0.094
<i>Other yeast</i>	5	1	4	1.000
<i>Aspergillus fumigatus</i>	5	3	2	0.194
<i>Other Aspergillus</i>	3	2	1	0.342
<i>Other mould</i>	3	1	2	1.000

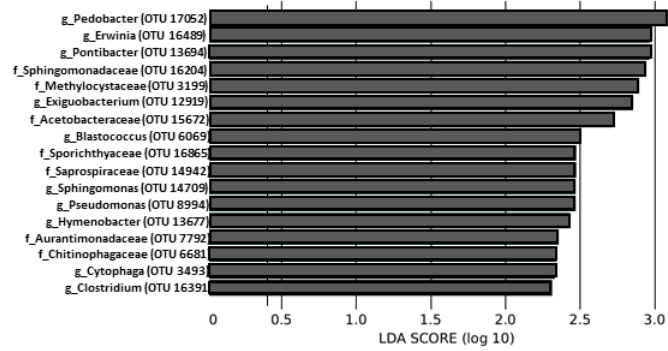
Indoor fungal contamination

Total fungal load (pg/EDC)	11.39 [9.82]	11.68 [12.76]	11.28 [8.98]	0.934
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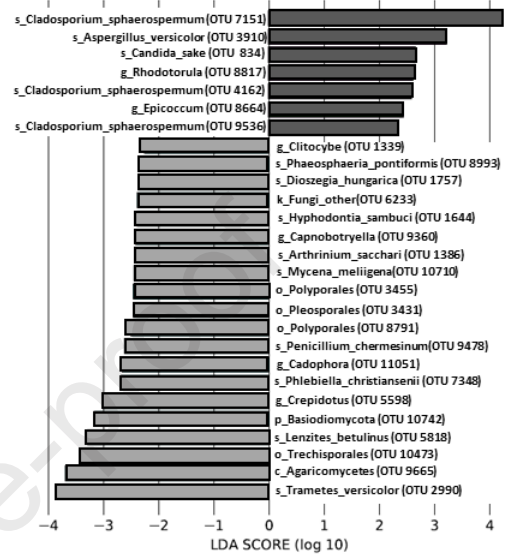
* p-value according to Man-Whitney test used for quantitative variables analysis and Fisher's exact test for qualitative variables. The values are presented as the mean [SD] or total count (%). **Asthma control as per the ACQ-7. ***ICS, Inhaled corticosteroid; LABA, Long acting beta2 agonist; LAMA, Long acting muscarinic antagonist; OCS, oral corticosteroid; [†]VRC, Voriconazole. ****NA: not available because all samples are in the same condition regarding these variables.



A



B



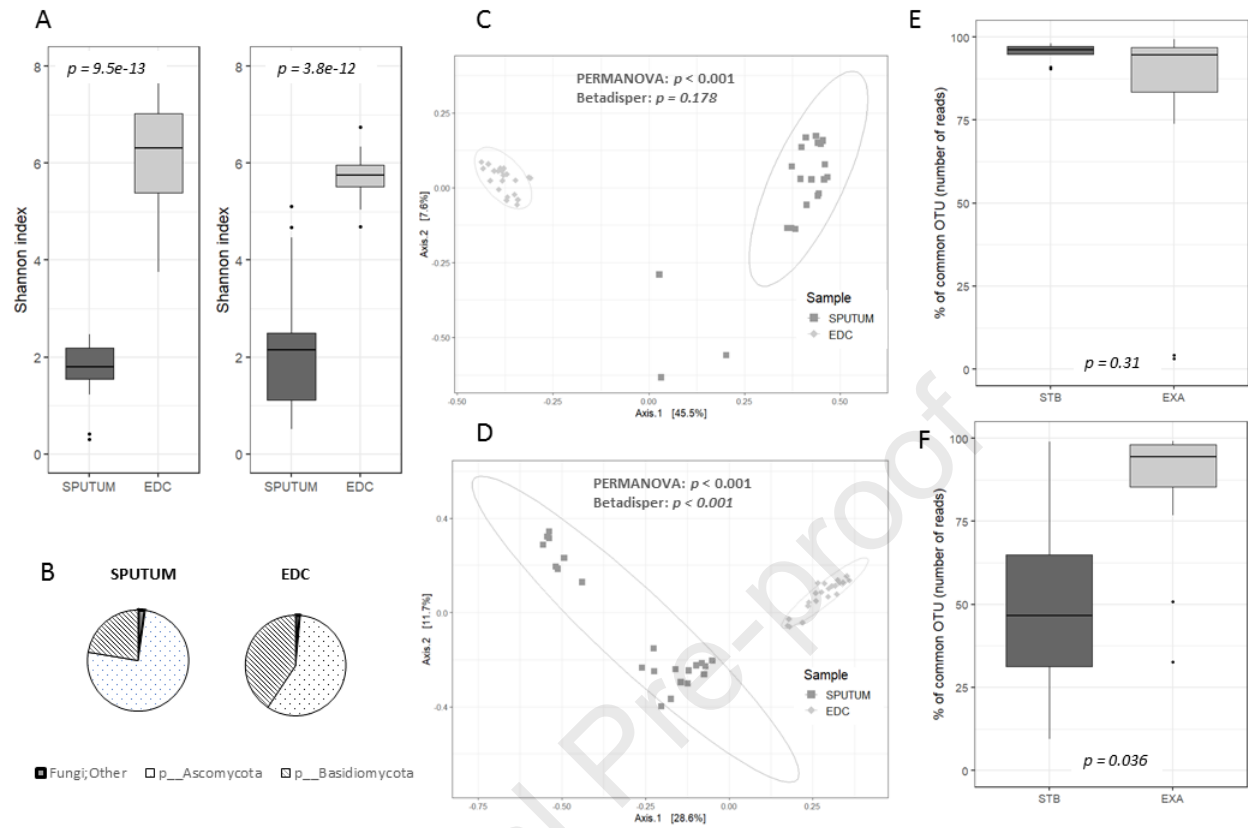
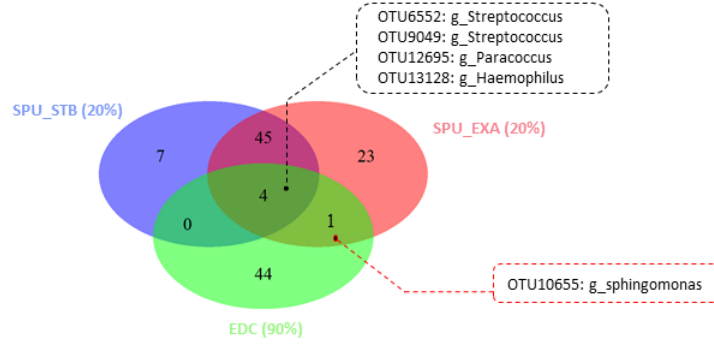


Figure 4.

A



B

