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Clinical relevance of *Aspergillus fumigatus* sensitization in cystic fibrosis

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Abstract

Rationale: The clinical relevance of sensitization to *Aspergillus (A) fumigatus* in cystic fibrosis (CF) is unclear. Some researchers propose that specific *A fumigatus* IgE is an innocent bystander, whereas others describe it as the major cause of TH-2-driven asthma-like disease.

Objectives: Lung function parameters in mild CF patients may be different in patients with and without *A fumigatus* sensitization. We aimed to ascertain whether allergen exposure to *A fumigatus* by bronchial allergen provocation (BAP) induces TH-2 inflammation comparable to an asthma-like disease.

Methods: A total of 35 patients, aged 14.8 ± 8.5 years, and 20 healthy controls were investigated prospectively. The patients were divided into two groups: group 1 (n = 18): specific (s)IgE negative, and group 2 (n = 17): sIgE positive (≥0.7 KU/L) for *A fumigatus*. Lung function, exhaled NO, and induced sputum were analysed. All sensitized patients with an FEV1 > 75% (n = 13) underwent BAP with *A fumigatus*, and cell counts, and the expression of IL-5, IL-13, INF-γ, and IL-8 as well as transcription factors T-bet, GATA-3, and FoxP3, were measured.

Results: Lung function parameters decreased significantly compared to controls, but not within the CF patient group. After BAP, 8 of 13 patients (61%) had a significant asthmatic response and increased eNO 24 hours later. In addition, marked TH-2-mediated inflammation involving eosinophils, IL-5, IL-13, and FoxP3 became apparent in induced sputum cells.

Conclusion: Our study demonstrated the clinical relevance of *A fumigatus* for the majority of sensitized CF patients. A distinct IgE/TH-2-dominated inflammation was found in induced sputum after *A fumigatus* exposure.

KEYWORDS

Aspergillus fumigatus, basic mechanisms, bronchial allergen provocation, clinical immunology, cystic fibrosis, IgE

1 | INTRODUCTION

Aspergillus (A) fumigatus is a ubiquitous fungal organism commonly found in house dust, water damaged walls or ceilings, and decomposing organic material. Its conidia are approximately 2-3.5 µm in diameter, which allows deposition in terminal airways and alveoli.[1] Several hundred *A fumigatus* conidia are inhaled by the human lung every day.[1] The host interaction with *A fumigatus* in cystic fibrosis (CF) lung disease is diverse in several aspects.[1-3] (a) The fungus chronically colonizes the CF airways, rarely triggering systemic, or invasive infections. (b) While 60% of adult patients are sensitized to *A fumigatus* with the presence of specific IgE and IgG, a subset of these adults mounts a robust allergic response with a high total IgE and substantially increased eosinophils.[4] (c) Only a small subset of those patients develops significant pulmonary symptoms, the full picture of bronchial pulmonary aspergillosis (ABPA).[3-5] The underlying mechanisms seem to be complex, and they are modulated by a variety of factors, including the cystic fibrosis transmembrane conductance regulator (CFTR), non-CFTR genetic immune host susceptibility and gene modifiers, patient's age and sex, atopy, microbial interactions, medication.[1-3] Early diagnosis of these different disease entities is critical in order to evaluate individual risk profiles and future treatment options, for example itraconazole, corticosteroids, and/or Vitamin D3. To date, the role and clinical relevance of specific IgE against *A fumigatus* are unclear. Some authors suggest that IgE is an innocent bystander, whereas others showed that neutralizing IgE with the monoclonal antibody omalizumab is effective in ABPA.[2,6,7] It can be hypothesized that the production of IgE and associated Th2-driven inflammation (IL-5, IL-4, IL-13) induce the transition from sensitization to *Aspergillus fumigatus* to an asthma-like condition leading to end-stage ABPA.[1,2,6] To test this hypothesis, patients with sensitization to *A fumigatus* were analysed by lung function and airway inflammation before and after bronchial allergen provocation (BAP) with lyophilized *A fumigatus* antigen. BAP is the gold standard to demonstrate the bronchial relevance of an allergen. Earlier studies from our working group showed that BAP is a safe and highly reproducible method in children and adults suffering from respiratory diseases.[3-6] Although great effort has been put into standardizing the procedure, BAP is still rarely used by clinicians due to possible side-effects and the fear of severe asthmatic reactions.[8-13] For safety reasons, only patients with mild CF (FEV1 > 75%) and proven sensitization to *A fumigatus* underwent BAP.

2 | MATERIALS AND METHODS

2.1 | Patients

Patients were recruited from the Division of Pediatric Pulmonology, Allergy and Cystic fibrosis, Goethe University, Frankfurt, Germany. The population of this study consisted of 35 clinically stable patients with CF (6 were *P aeruginosa*-infected), aged 4-41 years (average 14 years), and 20 non-smoking healthy control subjects. The CF patients were allocated to one of two groups: group 1 (n = 18): negative for sIgE against *A fumigatus*; and group 2 (n = 17): sIgE positive ≥ 0.7 KU/L, ≥ class 2.

Before inclusion in the study, a detailed verbal and written explanation took place with all patients. The course of the study, the goals, and the risks were discussed in detail with patients and/or their legal guardians. Prior to the start of the study, the subjects and/or their legal guardians signed the consent form. The study was approved by the Ethics Committee of the Goethe University, Frankfurt, and registered under the number ClinicalTrials.gov Identifier: NCT00906568. In order to characterize the patients, allocation was made to either of the two patient groups on the basis of the presence or absence of specific IgE (sIgE) to *A fumigatus*.

Exclusion criteria were as follows: other chronic diseases or infections (eg HIV, tuberculosis, and malignancy), pregnancy, therapy with systemic corticosteroids, permanent treatment with inhaled corticosteroids, documented alcohol and/or drug abuse, and inability to perform all study procedures. The study comprised three visits. While group 1 patients negative for sIgE to A *fumigatus* (sIgE negative) had one visit only, all patients of group 2 (sIgE positive) who underwent BAP had a second visit, followed by a third visit 24 hours later.

At visit 1, the following assessments were performed: medical history, clinical investigation, exhaled NO, lung function, methacholine testing, the collection of a throat swab for microbiological analysis, and a blood draw for measurement of total eosinophils and sIgE for *A fumigatus*. After conducting these examinations, sputum induction and sputum processing were performed.

At visit 2, group 2 (sIgE positive) patients with a FEV1 > 75% underwent BAP with *A fumigatus*. The patients received comprehensive information about the technique and completed FEV1 measurements for the next 12 hours. Our reasons for excluding sIgE-negative CF patients was not due to a decision of our ethic committee, but instead based on a literature research and our own experience. A literature review of all previous BAP studies showed that in sIgE-negative patients, BAP was always negative. In addition, BAP is time-consuming for the patients—at least 9 hours—and we therefore deemed it inconvenient and not ethical to perform BAP in sIgE-negative patients when we designed the study protocol.

At visit 3, 24 hours after BAP, a physical examination, measurement of exhaled NO, lung function testing, methacholine testing, and sputum induction was performed.

2.2 | Measurement of exhaled NO

Exhaled NO (eNO) was measured using the NIOX1 (Aerocrine, Solna, Sweden) according to American Thoracic Society guidelines.[14]

2.3 | Pulmonary function test

Pulmonary function tests were performed according to the recommendations of the American Thoracic Society and the European Respiratory Society.[15] The following measurements were obtained: FVC, FEV1, FEV1/VC, maximum FEV1 fall in early asthmatic reaction (EAR) and late asthmatic reaction (LAR), and PD20 FEV1-*A fumigatus*.

2.4 | Methacholine test

The methacholine test was performed using the Aerosol Provocation System (APS) (VIASYS Healthcare GmbH, Höchberg, Germany), as described previously in detail by our group.[16] During tidal breathing, the system determined the exact administered dose of methacholine automatically. Methacholine with a concentration of 16 mg/mL was inhaled in five steps: 0.01, 0.1, 0.4, 0.8, and 1.6 mg. The individual cumulative provocation dose (PD) causing a 20% drop in FEV1 (PD20FEV1) was calculated by logarithmic interpolation using an integrated programme.

2.5 | Bronchial allergen provocation

All patients were instructed not to take any medication for at least 24 hours before each BAP test. The BAP was performed in the morning, between 8:00 am and 12:30 pm using the flow-controlled nebulizer system APS (VIASYS Healthcare GmbH).[3] A Medic Aid nebulizer with a constant output of 160 mg/min was utilized, resulting in a particle size of 3.2 µm.[8] Continuous monitoring of the patient's breathing by the APS during the entire provocation ensured the inhalation of an exact amount of the allergen. For this purpose, an integrated flow sensor continuously measured the inspiratory flow and the inspiration time,[8] enabling the device to adapt the duration of inhalation to the amount of allergen to be inhaled.

For the allergen solution, lyophilized *A fumigatus* allergen (Allergopharma GmbH&Co.KG; Rheinbeck, Germany) was dissolved in 5 mL of a 0.9% saline solution, resulting in an allergen solution with a concentration of 5000 standardized biological units per ml (SBU/mL). All extracts from Allergopharma GmbH&Co.KG were standardized by in-house ELISA and by prick testing. The *A fumigatus* allergen was a non-modified native extract, completely free of endotoxins, and licensed in Germany for BAP, but no information on major allergen contents is available.

After the baseline FEV1 was determined, the patient inhaled a 0.9% saline solution without allergen. Two minutes later, the FEV1 was measured. In case of a decrease of more than 10%, the BAP test was postponed. Otherwise, the BAP test was conducted by stepwise inhalation of increasing amounts of the standardized allergen solution. Ten minutes after each step, spirometry was performed. The first step covered an inhalation of 5 SBU/mL. Afterwards, the dose was doubled until a decrease of FEV1 ≥ 20% was reached or a cumulative dose of 635 SBU/mL was administered.[8-10]

At the end of each test, every patient inhaled 2 puffs of salbutamol (200 µg) to improve the FEV1 to at least 80% of the baseline value. To detect late asthmatic response (LAR), FEV1 was measured every hour with the asthma monitor AM1® (VIASYS Healthcare GmbH) for up to 12 hours after BAP. Symptoms were recorded by the patients or their parents. The LAR was defined as a maximum FEV1 fall ≥ 15%. Patients were instructed to use salbutamol as rescue medication.

2.6 | Sputum collection and sputum cells

24 hours after BAP sputum was collected, patients first performed three baseline lung function tests according to ERS guidelines. Afterwards, they inhaled 400 µg salbutamol, and 20 minutes after administration, three more lung function tests were performed. Consecutively, nebulized hypertonic saline was administered at concentrations of 3%, 4%, and 5% every 7 minutes as described previously.[17-19] After each inhalation of the saline concentration, the mouth was flushed and the nose cleaned to decrease the amount of squamous epithelium cells within the samples. Sputum was processed within 2 hours of collection. The selected sputum plugs were picked as far as possible without saliva, processed into a weighed Eppendorf tube and processed with 4x weight/volume of 0.1% Dithiothreitol (DTT). Afterwards, 4× weight/volume of phosphate-buffered saline (PBS) was added. Samples were filtered through 48 µm mesh and centrifuged without breaks for 10 minutes at 790 × *g* to separate the supernatants, which were removed, and the samples were stored at −80°C until analysis. Four slides were generated from each sample for cellular differentiation. At least 400 inflammatory cells were counted for each specimen. Macrophages, neutrophils, eosinophils, and lymphocytes were expressed as percentages of the total cell count.[19,20]

2.7 | Extraction and transcription of mRNA and qRT-PCR

Total RNA from induced sputum (IS) cells was extracted using the innuPrep RNA Mini Kit (Analytic Jena, Jena, Germany), according to the manufacturer's instructions as described previously.[21] RNA quantification and quality assessments were performed by ultraviolet-visible spectrophotometry (Nanodrop Technologies, Wilmington, DE, USA). Also, the quality of RNA was checked by using the BioRad Experion (BioRad, Hercules, CA, USA), according to the MIQE guidelines.[22,23] The RNA quality of all samples reached an RNA integrity number between 8.9 and 9.7. Before reverse transcription, a DNase treatment was performed using DNase I (Qiagen, Hilden, Germany) to avoid DNA contamination. 150 nanograms (ng) of RNA in up to 4 µL RNase-free water were mixed with 4 µL 10xDNase buffer and 1 µL DNase I, then filled up to 10 µL with RNase-free water. Subsequently, incubation for 15 minutes took place. Finally, 1 µL of a mixture of 1 mL RNase-free EDTA 20 mmol/L added, followed by incubation at 65°C for 15 minutes in a thermocycler.

Reverse transcription of mRNA into cDNA was supplemented with a master mix of iScript Reverse Transcriptase (BioRad), performed as indicated by manufacturer's description and as previously described.[10]

Transcripts were quantified by performing a two-step real-time (RT) PCR with Eppendorf Mastercycler Realplex S detection system (Eppendorf, Hamburg-Eppendorf, Germany), using 96-well reaction plates (Greiner, Germany). In each well, a final concentration of

50 pg/mL of each specific primer pair, designed for real-time PCR and purchased from Sigma-Aldrich (Germany) and Qiagen (USA), was used in a 25 µL reaction vessel containing SYBR-Green Mastermix (Qiagen) RNAse-free water. The specific primers used in the real-time PCR are listed in Table S2. The amount of IL-5, IL-8, IL-13, INF-γ, T-bet, GATA-3, and FoxP3 mRNA expression was normalized with endogenous control GAPDH (ΔCt values), and the relative quantification and calculation of the range of confidence was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method (relative gene expression) as previously described.[24] All amplifications were carried out at least in duplicate.

2.8 | IgE Measurements

Total IgE and sIgE to *A fumigatus* were routinely determined according to the manufacturer´s instructions in our laboratory by chemiluminescence immunoassay (IMMULITE, Siemens Healthcare, Erlangen, Germany).[25]

2.9 | Statistical analysis

Data were analysed using the statistical program GraphPad Prism (version 5) and Microsoft Excel. The Mann-Whitney test was used for unpaired comparisons between patients (sIgE-negative vs. sIgE-positive patients) and controls. Paired samples (data before and after bronchial provocation) were analysed using the Wilcoxon rank-sum test. For non-normally distributed samples, the corresponding non-parametric tests were used. Spearman rank correlation was performed to test the relationship between the cumulative allergen dose and the specific IgE levels. Statistically significant differences were defined as *P* values $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

3 | RESULTS

3.1 | Characteristics of the study population

The population of this study consisted of 35 mild CF patients with and without sIgE to *A fumigatus* and 20 non-smoking healthy control subjects (see Table 1). It is important to note that sputum induction was not successful in all CF patients. Induced sputum was obtained from 13 to 18 (72.2%) patients without sensitization and 10 to 17 patients (58.8%) in the group with sensitization. The clinical characteristics of patients with and without successful sputum were comparable, as shown in Table S1. A significant decrease in FVC and FEV1 became noticeable compared with healthy control subjects (Table 1).

3.2 | Total and specific IgE levels

As shown in Table 1, there were significant differences for total IgE and sIgE between non-sensitized and sensitized patients.

3.3 | Methacholine testing

At baseline, methacholine testing showed no differences between sIgE-negative and sIgE-positive CF patients (1.3 + 1.3 mg vs. 1.7 + 0.3 mg methacholine)—see Table 1. Most patients had no

TABLE 1 Characteristics of patients and controls

	Control subjects	Patients (Total)	sIgE-negative group 1	sIgE-positive group 2
Number	20	35	18	17
Age (y)	17 ± 6.3	14.8 ± 8.5	14.1 ± 8.6	14.8 ± 8.7
Sex (m/w)	9/11	20/15	9/9	11/6
FVC (%)	99.4 ± 7.4	93.6 ± 12*	90.1 ± 7.31	97.2 ± 10.30
FEV1 (%)	101.0 ± 8.8	89.7 ± 19.1*	85.6 ± 12.5	93.2 ± 17.1
eNO (ppb)	14.9 ± 7.1	10.8 ± 6.1	10.2 ± 6.2	11.6 ± 6
Methacholine (mg)	n.d.	1.5 ± 0.8	1.3 ± 1.3	1.7 ± 0.3
Number of patients with > 1 mg methacholine (n)	n.d.	17 of 33	8 of 18	9 of 15
Total IgE (KU/L)	72.5 ± 44.2	173.5 ± 289.9	34 ± 33.3	321.1 ± 363.6**
sIgE *A fumigatus* (kU/L)	0.1 ± 0.1	11.1 ± 21.4	0.1 ± 0.04	22.7 ± 26.4**
Eosinophils (number per µL)	n.d.	190.2 ± 119	126.2 ± 89.8	256.9 ± 108.9
Pseudomonas colonization (n)	NA	6	2	4

Note: For all parameters, mean ± SD are shown; significant differences were found between all patients and controls for FVC and FEV1 ($^{*}P < .05$) and for total IgE and sIgE between the sIgE-negative and sIgE-positive patient group ($^{**}P < .01$).

Abbreviations: n.d., not done; NA, not applicable.

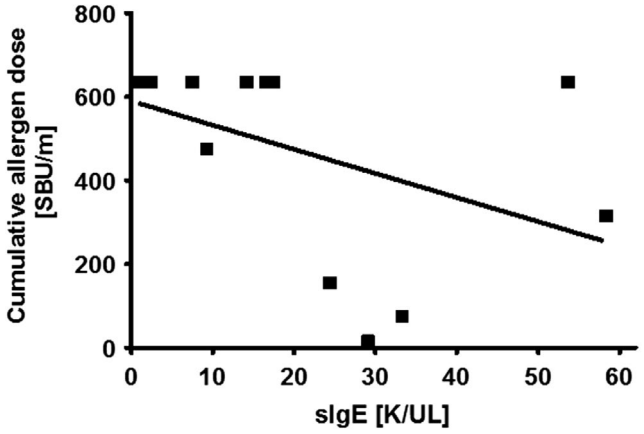

FIGURE 1 Correlation of sIgE *A fumigatus* to cumulative allergen dose. The Y-axis represents the cumulative allergen dose (SBU/ml), and the x-axis represents sIgE to Aspergillus (K/U/L). There was a significant correlation between sIgE and allergen doses of the BAP ($r = -0.579$, $P < .05$) Statistics: Spearman test

bronchial hyperreactivity at >1 mg methacholine (9 of 18 in the sIgE-negative vs. 9 of 15 in the IgE-positive patients). As expected, after BAP there was an increase in bronchial hyperreactivity (median before 1.79 mg/after BAP 1.22 mg methacholine). However, due to the small sample size, this was not significant.

3.4 | Bronchial Allergen Provocation leads to asthma-like symptoms

At visit 2, 13 from 17 (76.5%) of the sIgE-positive patients underwent BAP with *A fumigatus* since their lung function was above FEV1 > 75% according to the ATS guidelines.[26] Three patients showed an early asthmatic reaction (EAR), three patients had an EAR and a LAR, and two exhibited LAR only. Five patients showed no response. However, one patient who was considered "negative" due to the pre-defined cut-off level of 20% had a drop in the FEV1 of 19%. Interestingly, there was a significant correlation between sIgE quantification to cumulative BAP dose sensitivity ($r = -.579$; $P < .05$) in the Spearman correlation test, indicating that patients with high levels of sIgE are more likely to have a positive BAP than those with lower sIgE levels, as shown in Figure 1.

All patients with an EAR showed mild symptoms like cough and wheezing and were advised to use salbutamol. In addition, four of the five patients with an LAR used salbutamol for symptom relief. However, two of these patients exhibited a severe LAR with clinical symptoms such as shortness of breath, chest tightness, and cough. These two patients had a drop in FEV1 of 48% and 54%, respectively. Both were treated with salbutamol and oral steroids (prednisolone 50 mg). Lung function recovered completely within 24 hours, but patients showed a slight irritant cough for 3-4 days.

3.5 | Exhaled NO in sensitized and non-sensitized CF Patients

Baseline Exhaled NO (eNO) levels between sIgE-negative and sIgE-positive patients were invariant (Table 1). However, 24 hours after BAP, a significant eNO increase from 10.61 ppb ± 6.2 before and after BAP 16.2 ± 11.6 ($P < .05$) was recorded. In addition, patients showing an LAR had significantly higher eNO levels compared to patients with a negative BAP (25.26 ± 11.2 ppb vs. 7.92 ± 1.09 ppb; $P < .05$).

3.6 | Analysis of the inflammatory cell distribution in sputum

We analysed the inflammatory cell distribution in patient groups and healthy controls (Table 2). As mentioned before, acceptable sputum samples were obtained from 13 of 18 (72.2%) patients without sensitization (group 1), 10 of 17 patients (58.8%) in the group with sensitization (group 2) and all 20 healthy controls (100%). A significant increase was seen in neutrophils in all CF patients vs. controls at baseline (14.4% ±10.43; $P < .05$). No difference for eosinophils could be found (Table 2). Interestingly, 24 hours after BAP, a significant increase of eosinophils occurred (baseline: 1.38% ± 1.32; 24h after BAP: 8.14% ± 7.35).

3.7 | Expression of mRNAs

The mRNA expression of the pro-allergic TH-2 key cytokines IL-5 and IL-13, the TH-1 key cytokine IFN-γ, the pro-inflammatory chemotactic factor IL-8, and the TH-1 and TH-2 master transcription

TABLE 2 Sputum cell counts in induced sputum

Parameter	Controls	Patients (Total)	sIgE-negative group 1	sIgE-positive group 2	sIgE-positive 24 h after BAP
Number (n)	20	23	13	10	9
Macrophages (%)	92 ± 13.46	81.50* ± 5.85	82.17 ± 13.38	80.95 ± 6.84	78.44 ± 18.94
Neutrophils (%)	6 ± 8.03	14.40* ± 10.43	14.42 ± 13.44	14,39 ± 7.88	11.83 ± 20.66
Lymphocytes (%)	13 ± 6.88	2.45 ± 1.20	2.58 ± 1.20	2.33 ± 1.26	1.22 ± 0.89
Eosinophils (%)	1 ± 1.10	1.38 ± 1.32	0.78 ± 0.96	1.87 ± 1.45	8.14§,* ± 7.35

Note: At least 400 sputum cells were counted for each specimen. Macrophages, neutrophils, eosinophils, and lymphocytes were expressed as percentages of the total cell count. Mean ± SD are shown. A significant increase of neutrophils in CF patients and controls was found (*$P < .05$). 24 h after BAP, a significant increase of eosinophils (§,*$P < .05$) was found.

factors T-bet and GATA-3, as well as the master transcription factor of regulatory T cells FoxP3, were differentially regulated in sputum derived from CF patients compared with healthy controls (Table 3). As described before, the expression of the pro-inflammatory cytokine IL-8 was significantly elevated in all CF patients (median 4.12, range 1.98-10.4) compared with control subjects (median 1.0, range 0.32-1.72; $P < .001$). In addition, IFN-γ expression was significantly up-regulated in CF (median 7.18, range 2.88-10.91) compared with controls (1.0, 0.6-1.6; $P < .01$). The transcription factors T-bet (8.8-fold expression), GATA-3 (4.9-fold expression), and FoxP3 (2.9-fold expression) were significantly elevated compared with healthy controls. However, no differences were found for the TH-2 related cytokines IL-5 (1.9-fold expression) and IL-13 (1.7-fold expression; see Table 3). At baseline, no significant differences could be seen within the groups of sIgE-positive and sIgE-negative patients. In contrast to baseline findings, expression of TH-2 related cytokines IL-5 and IL-13, the TH-2 master transcription factor GATA-3, and the homeostatic transcription factor FoxP3 were distinctly elevated, indicating an asthma-like inflammation in the airway 24 hours after BAP with *A fumigatus* (Figure 2).

4 | DISCUSSION

The lung disease cystic fibrosis is characterized by perpetuating inflammation, recurrent infection, and mucus hypersecretion.[17,18,27] Among the major cell types within CF airways, after neutrophils both B and T lymphocytes are found in large numbers beneath the surface epithelium.[28,29] In infants with CF, exaggerated production of pro-inflammatory cytokines was observed in lower airways.[17,18,30] As many as 20-40% of CF patients have chronic airway colonization with *Aspergillus* species, an immediate skin reaction, and/or specific IgE/IgG antibodies to *A fumigatus*, which are detectable in up to 60% of patients during the course of the disease.[2,4,31] To shed some light on the unclear clinical relevance of airway sensitization with *A fumigatus*, we used induced sputum as a window to lung pathology, generating a local picture of the site of inflammation.[32] However, responses to *A fumigatus* seem to be of a diverse nature among different patients, and result in clinical deterioration only in a subset of patients. Beyond the full picture of an ABPA, the majority of patients are only sensitized to *A fumigatus*.[1] In the current study, we aimed to determine how this sensitization can be interpreted with regards to exposure to *A fumigatus* in the daily life of CF patients. It is currently unclear whether a subgroup of patients within the group of sensitized patients exist and whether that subgroup is more prone to exhibit an asthmatic reaction.

BAP was applied to characterize *A fumigatus*-specific immune responses.[11-13] As shown, *A fumigatus*-sensitized and non-sensitized CF patients did not exhibit differences in pulmonary function testing, for example FVC and FEV1, at baseline. Although mild and stable patients with a FEV1 > 75% were selected, CF patients lagged significantly behind the control group of healthy volunteers in terms of major lung function parameters. This finding confirms previous data that the majority of sensitized patients do not have a disadvantage by being sensitized to *A fumigatus*.[1,4,25,31] However, by looking at the sensitized patients in more detail by performing BAP, it was discovered that a large proportion of these patients showed an asthmatic reaction to *A fumigatus* accompanied by a significant drop in FEV1. Clinically responding patients showed wheezing and coughing, which is typical for an asthmatic reaction triggered by *A fumigatus*. Three of the patients had an EAR, three patients had EAR and LAR, and two patients showed an LAR only. Five of 13 patients were clinically tolerant to BAP. Overall, 61% of patients showed a significant reaction as a result of BAP. These data are in line with a study that demonstrated that even among weak skin reactions to *A fumigatus*, 43% are associated with a positive reaction in BAP.[33] Furthermore, the hypothesis that sensitization translates to clinical susceptibility like an asthmatic reaction was demonstrated by a significant correlation between sIgE sensitization (quantification) to cumulative BAP dose sensitivity. In addition, eNO significantly increased after 24 hours, and patients with an LAR had significantly higher eNO levels compared to patients with a negative BAP. This finding is well-known from studies in patients with allergic asthma.[10,34,35]

TABLE 3 mRNA expression changes in sputum cells

Target mRNA	Controls (n = 19)	Patients (Total) (n = 23)	sIgE-negative (n = 13)	sIgE-positive before BAP (n = 10)	sIgE-positive after BAP (n = 9)
IL-5	1.01 (0.63-2.36)	1.94 (0.95-5.47)	1.29 (1-3.12)	1.4 (0.3-4.24)	7.17 (3.7-10.67)**
IL-8	1.01 (0.32-1.72)	4.12*** (1.98-10.4)	3.35 (1.36-7)	3.69 (2.11-7.89)	2.63 (0.17-2.97)
IL-13	1.03 (0.62-1.59)	1.74 (0.1-3.84)	1.7 (0.12-2.53)	1.29 (0.02-2.15)	6.1 (3.53-9.98)**
INF-γ	1.04 (0.6-1.5)	7.18** (2.88-10.91)	6.61 (3.68-7.79)	6.29 (2.88-10.25)	2.87 (1.34-6)*
T-bet	1.01 (0.04-1.59)	8.72** (05-13.24)	8.87 (5.32-9.92)	8.21 (1.87-13.24)	1.27 (0.32-4.75)**
GATA-3	0.97 (0.48-1.81)	4.95** (1.05-9.92)	5.16 (1.8-9.92)	4.58 (2.63-7.79)	11.7 (7.5-13.39)**
FoxP3	1.03 (0.71-1.92)	2.99* (0.87-6.78)	3.76 (2.1-6.78)	2.49 (0.5-5.16)	7.82 (5.14-10.73)**

Note: mRNA expression fold changes (absolute values) of IFN-γ, IL-5, IL-8, and IL-13, as well as T-bet, FoxP3, and GATA-3 in sputum cells are shown. Data shown are medians and ranges. Significant differences (*$P < .05$, **$P < .01$, ***$P < .001$) were found between all patients and controls for IL-8, INF-γ, T-bet, GATA-3, and FoxP3 and between sIgE-negative and sIgE-positive patients for IL-5, IL-13, INF-γ, T-bet, GATA-3, and FoxP3.

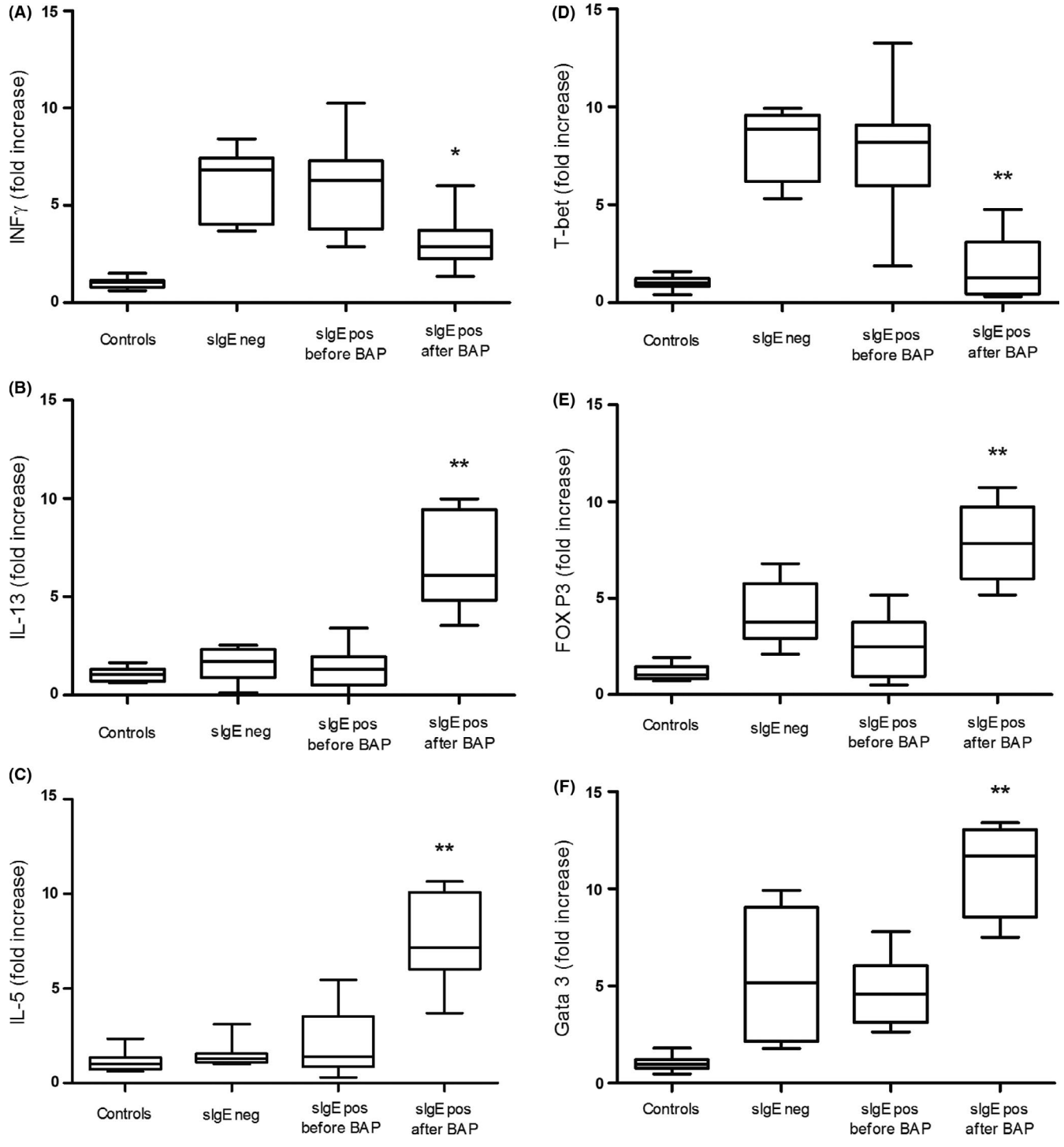

FIGURE 2 Changes in mRNA expression in the sputum of healthy control subjects and *A fumigatus* non-sensitized and sensitized patients. For sensitized patients, values before and 24 h after BAP are depicted. mRNA expression fold changes (absolute values) of IFN-γ, IL-5, and IL-13, (A-C) T-bet, FoxP3, and GATA-3 (D-F) in sputum cells are shown on the Y-axis. Data shown are medians and ranges. Significant differences between the groups are shown. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

As expected, bronchial hyperactivity measured by methacholine slightly increased without reaching significance, most likely due to the small number of patients who underwent BAP. However, clinical symptoms, degree of sensitization, lung function, eNO, and salbutamol use clearly demonstrate that *A fumigatus* exposure induced an asthma-like reaction in the majority of sensitized CF patients.

The strength of our findings is that the asthmatic reaction to *A fumigatus* was also reflected in the increase of important TH-2 biomarkers. Sputum eosinophils, IL-5, and IL-13 were increased significantly.[36,37] The effect of *A fumigatus* exposure to cause an imbalance of the immune homeostasis of TH-1 and TH-2 cytokines was further supported by the significant up-regulated expression of GATA-3 and

FoxP3. The significant increase of IL-5, IL-13, transcription factors, and eosinophils as well as the inverse decrease of IFN-γ and T-bet described in this study were also observed in previous publications of segmental allergen challenges in asthmatics and CF mouse models.[36-40] Both IL-5 and IL-13 are produced by TH-2 cells and are key activators of eosinophils and IgE production. In this context, repeated *A fumigatus* exposure in sensitized patients may lead to massive accumulation of eosinophils and activated TH-2 cells in the bronchial lumen that results in bronchial impaction, a hallmark of severe asthma and ABPA.[41,42] Considerable evidence indicates that IgE sensitization to *A fumigatus* and/or colonialization of the respiratory tract by *A fumigatus* in asthmatic patients is associated with reduced lung function and severe disease.[2,43,44] The important role of sIgE in response to *A fumigatus* is further highlighted by several clinical studies that show omalizumab as an interesting therapeutic strategy in ABPA, since it is associated with fewer side-effects compared to long-term corticosteroids.[7,45] In addition, we showed that BAP with *A fumigatus* was completely negative in patients under treatment with omalizumab.[45]

Unfortunately, neither the TH-2 cytokines nor the transcription factor pattern could replace the diagnostic value of BAP. Sensitization to *A fumigatus* is therefore initially a laboratory or skin test finding, which together with sIgE to *A fumigatus* might be a possible justification for BAP as diagnostic test.[12,46] Future studies may generate data from sufficient patient numbers to determine a certain *A fumigatus* sIgE cut-off level for CF patients. The use of recombinant allergens helped in the diagnosis of ABPA in scientific studies, but not in answering questions about clinical response in a real-life setting. The BAP strengthens the clinical diagnosis and supports the patient in preventing an asthmatic reaction upon exposure and provides direction for acute therapeutic strategies.

However, this study has some limitations. The number of CF patients who underwent BAP was very small. There were several reasons for this. According to ATS guidelines, only patients with a lung function > 75% could be challenged, because the risk of severe side-effects has to be considered. In addition, even in our CF community, there was great fear of severe side-effects. This was the major reason why some patients did not give their consent for the study and why our sample of patients with BAP was relatively small.

The study could also be criticized due to the fact that patients without sIgE were not challenged as a control group to rule out nonspecific toxicity or even contaminants in the *A. fumigatus* extract. BAP is a long-lasting procedure and patients have to be monitored carefully for 9 hours; therefore, we found it intrusive and not ethical to subject sIgE-negative patients to BAP. In addition, the *A fumigatus* used in the study was endotoxin free, and no pro-inflammatory signals, such as an increase of IL-8 or T-bet, were detected in sputum after BAP.

It would have been nice to be able to measure Aspergillus IgG or its relevant recombinant epitopes. However, this was not done, since that was assay is not available at our university, and sending it to an external laboratory was cost-prohibitive without a sponsor or other source of funding.

In conclusion, BAP with *A fumigatus* caused both a significant decrease in FEV1 in the majority of sIgE-positive patients as well as asthma-like symptoms with increased salbutamol use. A marked TH-2 mediated inflammation involving eosinophils, IL-5, IL-13, FoxP3, and eNO was demonstrated. Current clinical practice should be aware of the possible clinical relevance of *A fumigatus*-induced asthma-like disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data from this study are available on request from the Department for Children and Adolescents, Division of Allergology, Pulmonology and Cystic fibrosis, Goethe University, Frankfurt.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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