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Original Article

# Airway inflammation in mild cystic fibrosis☆

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**Abstract**

*Background:* Airway infection and inflammation play major roles in the progression of cystic fibrosis (CF) lung disease. In patients with mild disease, airway inflammation is a clinically relevant and often underdiagnosed feature. Lung function, sputum cell counts, and cytokine profiles in CF with mild disease might be different in patients with and without involvement of small airway disease (SAD).
*Methods:* Patients with mild CF ($n$ = 32) and 22 healthy controls were enrolled in this study. Patients with CF were assigned to two groups: (1) patients without SAD ($n$ = 19, median age 12.3 years, $MEF_{25}$ > 50% predicted), and (2) patients with SAD ($n$ = 13 median age, 13.2 years, $MEF_{25}$ < 50% predicted). Lung function parameters were measured, cells in induced sputum were counted, and cytokines/chemokines (IL-1β, IL-6, IL-8, TNF-α) were analyzed by real-time quantitative PCR (qRT-PCR) and cytometric bead array (CBA).
*Results:* Patients with CF had significant elevated levels of pro-inflammatory genes in qRT-PCR and secreted gene products in CBA compared to controls. Patients with CF and SAD had significantly increased trapped air (RV/TLC) and pronounced airway inflammation compared to controls as indicated by elevated levels of sputum biomarkers like total cells, neutrophils, and IL6.
*Conclusions:* Our study demonstrated that patients with CF with mild disease defined by lung function might be further endotyped according to their involvement of SAD. In patients with CF and SAD, airway neutrophilic inflammation is more pronounced and is in part distinct from that seen in patients without SAD.


*Keywords:* Cystic fibrosis; Small airway disease; Airway inflammation; Pro-inflammatory biomarkers; Induced sputum; Gene expression

## 1. Introduction

In cystic fibrosis (CF), progressive pulmonary disease is the major cause of morbidity and mortality. The pathologic processes in CF, which are caused by a progressive inflammatory response with elements of tissue remodeling, airway obstruction and reduction in expiratory flow rates, ultimately lead to death from respiratory failure [1]. Notably, airway inflammation may already be present in young children with CF even in the absence of infection. Increased pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, as well as neutrophilic influx are prominent early features of CF inflammation [2]. Pulmonary function significantly correlates with induced sputum measurements of IL-8, neutrophil elastase, total cell counts, and neutrophil counts [3,4]. This inflammatory response has been attributed to activated NFκB, which is most likely intrinsically upregulated by mutations in

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the gene that encodes the CF transmembrane conductance regulator (CFTR) gene [5].

Although the importance of infection and inflammation in the pathophysiology of CF is well established, few data measuring airway inflammation in patients with mild CF are available. Tiddens and colleagues reviewed the important role of small airways in the pathophysiology of early CF lung disease [6]. Small airways (airways $<2$ mm/cm$^2$) are defined as the membranous bronchioles that do not take part in gas exchange. In CF, as in chronic obstructive lung disease (COPD) and asthma, especially small airways are thickened in relation to the severity of the airway inflammation [6]. Early closure of the smallest airways, probably due to neutrophilic inflammation, is diagnosed by assessment of lung function showing peripheral airway obstruction ($MEF_{25}$), raised residual volume to total lung capacity (RV/TLC) or increased lung clearance index (LCI) [7,8]. Studies based on computed tomography scanning [9] have confirmed the central role of small airways in cystic fibrosis. Therefore, we believe that a better characterization of inflammatory profiles in the individual CF patient could lead to a more targeted and more effective therapy. The sputum induction compared to bronchoalveolar lavage provides an easy to perform non-invasive method to get a detailed insight into patient-specific inflammation patterns.

In our study, differential cell counts, gene expression, and protein measurements were used to characterize airway inflammation in induced sputum of patients with mild cystic fibrosis with and without SAD and healthy control subjects. Since we could recently show that the pro-inflammatory cytokines (IL-1ß, IL-6, IL-8, and TNF-α) were significantly elevated in induced sputum of patients with moderate to severe CF, we selected the same biomarkers for patients with mild lung impairment [4].

## 2. Materials and methods

### 2.1. Subjects and selection

Thirty-six mild patients with CF and almost preserved lung function, forced vital capacity (FVC) > 75% predicted were recruited from the outpatient Christiane Herzog CF Center, Johann Wolfgang Goethe-University, Frankfurt, Germany. Four patients with CF were excluded since their $FEV_1$ was below 70% of that predicted. Control subjects were recruited from friends of the CF subjects and though advertising in the Frankfurt area. After informed consent, patients with CF and control subjects performed lung function testing and induced sputum was collected. Sputum samples were harvested after salbutamol inhalation. The collected sputum was processed and stored until analysis by certified laboratory personnel, blinded to the clinical findings.

Clinical stable patients with CF were assigned to two groups according to their efforts in lung function testing: patients without SAD with $MEF_{25} > 50\%$ predicted ($n = 19$) and patients with SAD with $MEF_{25} < 50\%$ predicted ($n = 13$). Exclusion criteria were FVC < 75% predicted, $FEV_1 < 70\%$ predicted, acute lung infection four weeks before inclusion, therapy with systemic corticosteroids, treatment with inhaled corticosteroids 14 days before inclusion, and inability to perform lung function tests. Clinical stability was defined as absence of acute exacerbation of disease 4 weeks prior to inclusion. Acute exacerbation was defined by two of the following symptoms: Fever >38.0 °C, increase of sputum, significant increase of C-reactive protein (CrP) and significant weight loss.

### 2.2. Ethics, consent, and permissions

Human guidelines of good clinical practice and the declaration of Helsinki (1964), and Edinburgh (2000) were followed in the conduct of the trial. The study was approved by the Ethics Committee of University Hospital Frankfurt am Main and registered at clinicaltrials.gov (NCT00906568). All the parents and all patients older than sixteen years of age provided written informed consent.

### 2.3. Lung function tests

Baseline lung function was evaluated using a body plethysmograph by VIASYS Healthcare GmbH (Hoechberg, Germany). The following parameters were recorded: FVC, $FEV_1$, Tiffeneau index ($FEV_1$/FVC), $MEF_{25}$, residual volume (RV), total lung capacity (TLC), and trapped air (RV/TLC).

### 2.4. Sputum collection and processing

Subjects first inhaled 400 µg salbutamol followed by threefold inhalation of hypertonic saline at concentrations of 3%, 4%, and 5% every 7 min consecutively as recently described [4]. During this procedure, it was important to flush and clean the mouth to minimize the amount of squamous epithelium cells in the samples. Sputum was processed within one hour of collection. The selected sputum plugs contained as little saliva as possible. The sputum samples were placed in a weighed 50 mL Falcon tube (BD Biosciences, Germany) and processed with 4:1 (weight to volume) of 0.1% dithiothreitol (DTT). Afterwards, 2:1 (weight to volume) of phosphate-buffered saline (PBS) was added. Samples were filtered through 70 µm mesh and centrifuged for 10 min at $790 \times g$ to remove the cells. Supernatants were stored at −80 °C until further analysis with a protein assay.

### 2.5. Sputum cells

Specimens in which less than 10% of the inflammatory cells were squamous epithelial cells were considered adequate. At least 400 inflammatory cells were counted for each specimen. Neutrophils, lymphocytes, eosinophils, and macrophages were expressed as cells per mL and as percentages of the total cell count.

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### 2.6. RNA extraction

Total RNA from induced sputum was extracted using the innuPrep RNA Mini Kit (Analytic Jena, Jena, Germany) according to the manufacturer's instructions. All sputum plugs, at least 80 mg, were processed with 0.1% DTT and PBS/BSA and were processed according to the manufacturer's instructions. Then, 5 μL of RNA was diluted 1:5 (v:v) with RNase-free water, and the absorbance was measured to determine the amount of RNA. Also, the RNA quality was controlled using the BioRad Experion (BioRad, Hercules, CA, USA), according to the MIQE guidelines [10]. Before reverse transcription, a DNase treatment was performed using DNase I (Qiagen, Hilden, Germany).

### 2.7. mRNA reverse transcription

The processed RNA samples were supplemented with 9 μL of a master mix of 1 μL iScript Reverse Transcriptase (BioRad, Hercules, CA, USA), a random hexamer and oligo-dT mix, 4 μL 10× iScript RT buffer and 4 μL nuclease-free water. Afterwards, samples were incubated in a thermocycler at 25 °C for 5 min for an initial incubation step, then at 42 °C for 30 min and finally at 85 °C for 5 min.

### 2.8. Real-time qRT-PCR

Transcripts were quantified using two-step real-time RT-PCR with an Eppendorf Mastercycler realplex S detection system (Eppendorf, Hamburg-Eppendorf, Germany) in Greiner 25 μL 96-well reaction plates (Greiner, Germany). The amount of IL1ß, IL6, IL8, and TNFα mRNA expression was normalized with endogenous control glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and the relative quantification and calculation of range of confidence was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method (relative gene expression), as previously described [11]. All amplifications were carried out at least in duplicate. Data expression and statistical analysis of genes involved in immune cells and inflammation markers were analyzed as described [11].

### 2.9. Protein assay

Concentrations of six different cytokines/chemokines were determined in sputum samples by BD™ CBA System (BD Bioscience-PharMingen, San Diego, CA, USA) for the measurement of IL-1ß, IL-6, IL-8, and TNF-α levels. Each BD™ CBA Set contained one bead population with distinct fluorescence intensity, as well as the appropriate phycoerythrin (PE) detection reagent and standard. The tests were performed according to the manufacturer's advice, and samples were run in duplicate.

### 2.10. Statistical analysis

Data were analyzed using the statistical program GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) and Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Group differences between CF patients without and with SAD and healthy controls were analyzed using a Kruskal–Wallis test or a Mann–Whitney test depending on Gaussian distribution and the homogeneity of the variances. A probability of $p < 0.05$ was regarded as significant.

## 3. Results

### 3.1. Characteristics of the study population

Patients with CF were assigned to two groups according to their involvement of small airways. One group consisted of patients without SAD, expressed by $MEF_{25} > 50\%$ predicted ($n = 19$), while the other group consisted of patients with SAD expressed by $MEF_{25} < 50\%$ predicted ($n = 13$). Both CF groups were defined by well-matched clinical features regarding sex distribution, age at diagnosis, number of patients homozygous for F508del, body mass index (BMI), and colonization with *Pseudomonas aeruginosa*. We also included an age-matched healthy control group. The clinical characteristics of all groups are summarized in Table 1. Patients with CF and SAD showed significantly impaired lung function parameters mirrored by a reduction in FVC, FEV1, $MEF_{25}$, and RV/TLC compared to healthy control subjects as well as to CF patients without SAD (Fig. 1 and Table 1). Notably, patients with CF but without SAD showed no significant differences for FVC, $FEV_1$, $MEF_{25}$, and RV/TLC compared to healthy controls.

### 3.2. Increased inflammation in patients with CF and SAD

Sputum cell counts of the entire CF group differed from healthy controls in total, differential cell counts, especially neutrophils, and cell burden (Table 2). CF sputum had a median total count of $4.5 \times 10^6$ cells (healthy controls median: $2.85 \times 10^6$ cells), of which 45% were neutrophils (healthy controls median percentage: 1.75%), 64.63% macrophages (healthy controls median percentage: 97%) and 1.05% lymphocytes (healthy controls median percentage: 3.3%). The cell burden was significantly higher in the entire CF group ($86 \times 10^4$ cells/mL) than in the control group ($22.50 \times 10^4$ cells/mL). Comparing the two groups of patients with CF, the patients with SAD had significantly increased total cell numbers and neutrophil counts. Sputum from patients with CF and SAD, in comparison with CF patients without SAD, had a higher median total cell count ($8.5 \times 10^6$ cells versus $2.4 \times 10^6$ cells, $p < 0.05$), a higher percentage of neutrophils (median percent, 74.5 versus 19.5, $p < 0.05$), and lower percentage of macrophages (median percent, 24 versus 79.5, $P < 0.01$). In contrast, the number of alveolar macrophages in patients with CF and SAD was significantly decreased compared to controls and patients without SAD. (See Table 2.)

### 3.3. Gene expression levels of selected biomarkers in induced sputum of patients with CF indicate involvement of small airways

Levels of the pro-inflammatory biomarkers IL1ß, IL6, IL8, and TNFα were investigated using gene expression analysis

Table 1
Patient characteristics.

| | Controls, $n = 22$ | CF, $n = 32$ | CF without SAD, $n = 19$ | CF with SAD, $n = 13$ | $p$-value, CF without SAD vs. CF with SAD |
|---|---|---|---|---|---|
| Age | 16.2 (7.8–24.5) | 12.9 (7.4–55.0) | 12.3 (7.8–31.7) | 13.2 (7.4–55.0) | n.s. |
| Sex (m/f) | 11/11 | 18/14 | 12/7 | 6/7 | n.s. |
| F508del homozygous, $n$ (%) | n.d. | 14 (43.8) | 9 (47.4) | 5 (38.5) | n.s. |
| FVC (%) | 101.7 (83.4–113.6) | 95.0* (75.0–120.7) | 96.0 (77.5–120.7) | 94.2* (75.0–108.6) | n.s. |
| $FEV_1$ (%) | 106.3 (83.8–121.6) | 93.6** (70.0–119.4) | 96.2 (88.6–119.4) | 81.6*** (70.0–100.3) | <0.0001 |
| Bronchiectasis, $n$ (%) | n.d. | 30 (93.8) | 17 (89.5) | 13 (100) | n.s. |
| CrP (mg/dL) | n.d. | 0.08 (0.01–2.84) | 0.05 (0.01–1.16) | 0.1 (0.04–2.84) | n.s. |
| IgG (mg/dL) | n.d. | 968 (621–1774) | 1018 (621–1424) | 968 (741–1774) | n.s. |
| Ps.a, $n$ (%) | n.d. | 6 (18.8) | 2 (10.5) | 4 (30.8) | n.s. |
| S.a., $n$ (%) | n.d. | 25 (78.1) | 16 (84.2) | 9 (69.2) | n.s. |
| Other, $n$ (%) | n.d. | 18 (56.3) | 12 (63.2) | 6 (46.2) | n.s. |

Ps. a., *Pseudomonas aeruginosa* colonization, S.a., *Staphylococcus aureus* colonization, other: *Streptococcus pneumoniae* (1), *Streptococcus agalactiae* (1), *Sphingomonas paucimobilis* (1), *Stenotrophomonas maltophilia* (1), *Haemophilus influenzae* (1), *Haemophilus parainfluenzae* (1), *Klebsiella pneumoniae* (1), *Aspergillus fumigatus* (3), and *Candida albicans* (8). Data shown as median (range). The non-parametric Mann–Whitney test was used for comparisons between CF patients without and with SAD and healthy controls. Asterisks indicate significant differences between groups compared to healthy controls. Differences between CF groups and controls: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

and cytometric bead array in sputum cells and supernatants, respectively. We found significantly increased levels of IL1ß, IL6, and IL8, but not of TNFα, in sputum samples of patients with CF compared to the healthy control subjects (Fig. 2). We also found increased protein levels of IL-1ß and IL-8 in the sputum of both subgroups of CF patients compared to healthy controls (Fig. 3a, c). In contrast to the findings on gene expression level, no significant differences were found between the CF patients with and without SAD (Fig. 3).

The results from our study allow us to discriminate CF subgroups with and without affection of the small airways using sputum parameters like cell load, differential neutrophil counts as well as gene expression levels of pro-inflammatory cytokines.

## 4. Discussion

Controlling airway infection and excessive airway inflammation is an important issue in the treatment of CF lung disease [12,13]. A marked improvement of overall survival has been attributed to better patient care and aggressive treatment of infections, especially early eradication of *P. aeruginosa* infection [14]. Multiple factors have been proposed to explain the heterogeneity of disease severity in CF patients. These factors include residual activity of some CFTR mutations, the influence of other ion channels such as ßENaC [15], and differences in innate immune system response in the airways [16]. Furthermore, poor patient's compliance regarding treatment, lack of diagnosis of associated conditions, and biological resistance to therapy due to increased inflammatory responses in individual patients may contribute to a severe clinical course of disease [17,18].

Due to great efforts in patient care, pediatricians often see patients with mild disease and almost preserved lung function. The involvement of small airways plays an important role in the pathophysiology of CF lung disease, because small airways are especially thickened in relation to the severity of the airway inflammation [6]. Therefore, these mild patients with CF can be further phenotyped by means of lung function according to the presence or absence of SAD [6]. Early occlusion of the smallest airways, perhaps due to neutrophilic inflammation, may lead to peripheral airway obstruction and raised RV/TLC [7]. As a consequence, it is reasonable to anticipate that the inflammatory process in mild CF with SAD shows a different pattern of cellular inflammation and cytokines compared to the pattern in mild CF patients without SAD.

To address this issue, we recruited patients with mild CF with and without SAD, distinguished by $MEF_{25}$ greater or smaller than 50% predicted, and compared lung function data with clinical characteristics and several pro-inflammatory sputum biomarkers, including induced sputum inflammatory cells and cytokines/chemokines. As expected, RV and RV/TLC, which have been used as parameters to characterize air trapping, differed significantly between the groups.

In addition, the pattern of inflammation in the sputum differed significantly between the groups of CF patients. Subjects with SAD had a greater proportion of total cells and neutrophils in induced sputum than subjects without SAD,

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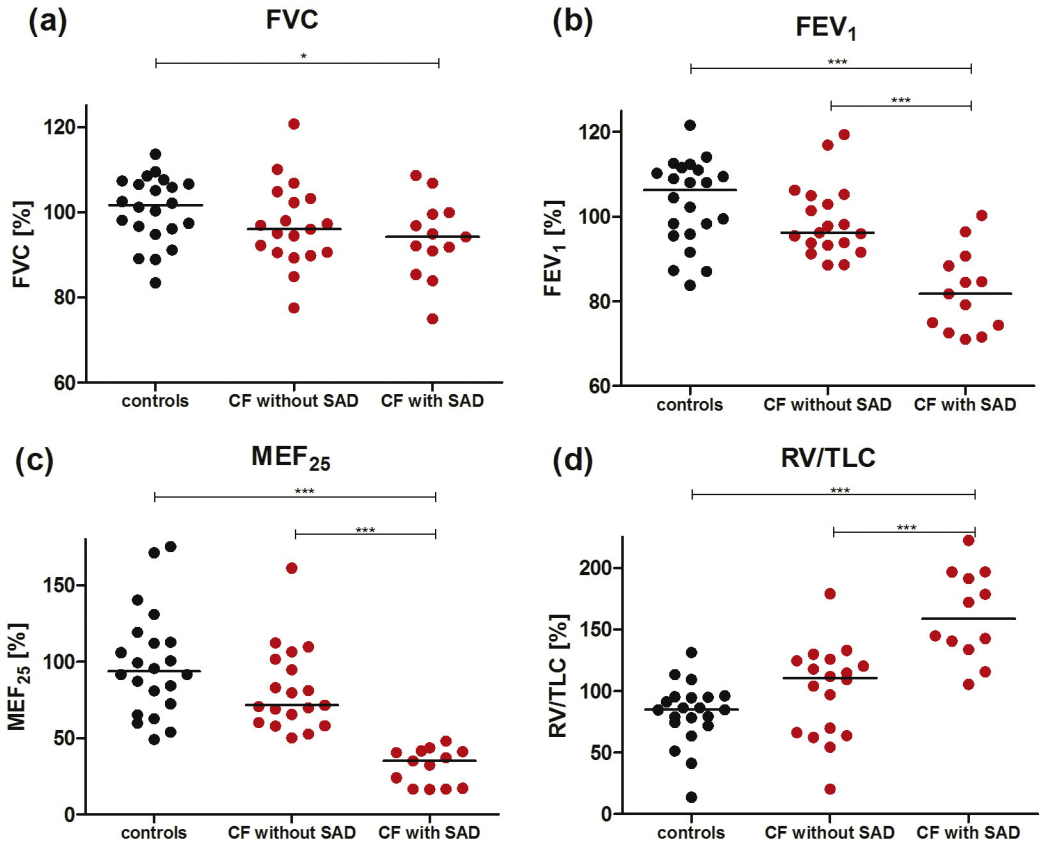


Fig. 1. Lung function measurements of CF patients and controls. Lung function data for (a) FVC, (b) $FEV_1$, (c) $MEF_{25}$, and (d) RV/TLC are shown as % predicted with the bar marking the median. The non-parametric Mann–Whitney test was used for comparisons between CF patients with and without SAD and healthy controls. Differences between CF groups and controls: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Table 2
Anti-obstructive and anti-inflammatory treatment.

| | Controls, $n = 22$ | CF, $n = 32$ | CF without SAD, $n = 19$ | CF with SAD, $n = 13$ | $p$-value, CF without SAD vs. CF with SAD |
|---|---|---|---|---|---|
| SABA, $n$ (%) | 0 | 12 (37.5) | 7 (36.8) | 5 (38.5) | n.s. |
| LABA, $n$ (%) | 0 | 16 (50.0) | 7 (36.8) | 9 (69.2) | n.s. |
| SAMA, $n$ (%) | 0 | 4 (12.5) | 0 | 4 (30.8) | $p < 0.05$ |
| LAMA, $n$ (%) | 0 | 2 (6) | 0 | 2 (15.4) | n.s. |
| LTRA, $n$ (%) | 0 | 3 (9.4) | 3 (15,8) | 0 | n.s. |
| AZM, $n$ (%) | 0 | 10 (31.3) | 4 (21.1) | 6 (46.2) | n.s. |
| DNAse, $n$ (%) | 0 | 16 (50.0) | 8 (42.1) | 8 (61.5) | n.s. |
| HS $n$ (%) | 0 | 12 (37.5) | 5 (26.3) | 7 (53.8) | n.s. |

SABA, short-acting ß-agonists; LABA, long-acting ß-agonists; SAMA, short-acting muscarinic antagonists; LAMA, long-acting antimuscarinics; LTRA, leuktoriene receptor antagonists; AZM, azithromycin; HS, hypertonic saline. Data are shown as absolute number of patients (percentage). The Fisher exact test was used for comparisons between CF patients with and without SAD.

consistent with other disease reports stating that airway inflammation starts mainly in the small airways. Notably, pro-inflammatory biomarkers like IL-1ß, IL-6, and IL-8 measured by RT-PCR and CBA were significantly increased in patients with mild CF compared to controls. Regarding IL1ß and IL8, this finding was independent from the involvement of the small airways. No marker, with the exception of IL6 mRNA level, showed significant differences when comparing the SAD group with patients without SAD. This might be explained in part because sputum is not exclusively harvested from the small but from the central airways, which is one important disadvantage compared to BAL. Furthermore, low concentrations of IL-6 protein in CF were described by others [19], whereas the discrepancy between IL6 gene expression and protein measurement in sputum is difficult to explain. One hypothesis is that IL-6 is rapidly degraded by local proteases. However, spiking and incubation experiments of IL-6 with proteases in vitro did not confirm this hypothesis [20]. Alternatively, IL-6 is rapidly bound to its receptor and IL-6 protein can only be detected in patients with more pronounced disease.

IL-8 acts as potent chemoattractant for neutrophils [16] and may have accounted for the excess of neutrophils that were seen even in mild patients without SAD compared to controls. Not surprisingly, as has been reported by others [21], high levels of IL-8 may be attributed to significantly upregulated

Table 3
Sputum cell counts in induced sputum of CF patients and healthy controls.

| | Controls, $n = 22$ | CF, $n = 32$ | CF without SAD, $n = 19$ | CF with SAD, $n = 13$ | $p$-value, CF patients with SAD vs. CF patients without SAD |
|---|---|---|---|---|---|
| Total cells ($\times 10^6$) | 2.85 (0.7–33.9) | 4.50 (0.3–114.6) | 2.40 (0.60–59.2) | 8.50** (0.3–114.6) | $p < 0.05$ |
| Cells/mL ($\times 10^4$) | 22.50 (6.5–153.0) | 86.00*** (14.0–888.0) | 70.00*** (14.0–554.0) | 153.30*** (27.0–888.0) | |
| Macrophages (%) | 97.00 (62.8–100.0) | 64,63*** (6.3–93.3) | 79.50*** (18.0–93.3) | 24.00*** (6.3–80.0) | $p < 0.01$ |
| Neutrophil cells (%) | 1.75 (0.0–32.0) | 45.00*** (4.0–92.8) | 19.50*** (3.0–81.0) | 74.50*** (4.0–92.8) | $p < 0.05$ |
| Eosinophil cells (%) | 0.00 (0.0–7.8) | 0.00 (0.0–6.5) | 0.00 (0.0–2.0) | 1.00* (0.0–6.5) | |
| Lymphocytes (%) | 3.30 (0.0–36.0) | 1.05 (0.0–8.4) | 1.63 (0.0–8.4) | 1.00 (0.0–6.5) | |

Data are shown as median (range).The non-parametric Mann–Whitney test was used for comparisons between CF patients without and with SAD and healthy controls. Asterisks indicate significant differences between groups compared to healthy controls. Differences between CF groups and controls: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

NFκB expression. NFκB-mediated chronic inflammation is a prominent feature even in mild lung disease, and whereas the mechanism by which CFTR regulates these inflammatory signaling pathways is critical, the exact mechanisms are still not known. Recent data suggest that CFTR downregulates NFκB and IL8 promoter activities in CF cell lines [22]. In addition,

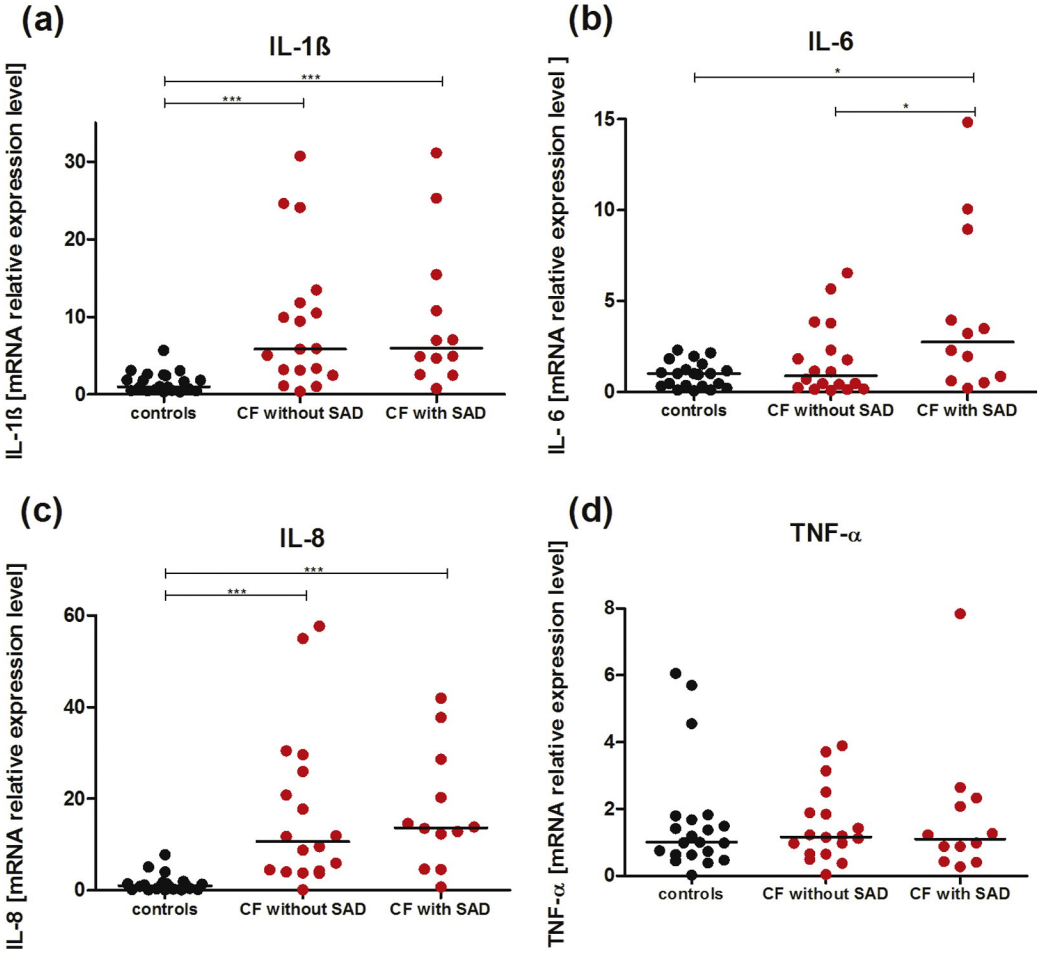


Fig. 2. mRNA expression levels for selected cytokines/chemokines in sputum of CF patients and controls. Data for (a) IL-1ß, (b) IL-6, (c) IL-8, and (d) TNF-α are shown as relative gene expression using comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method with the bar marking the median. The non-parametric Mann–Whitney test was used for comparisons between CF patients with and without SAD and healthy controls. Differences between CF groups and controls: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

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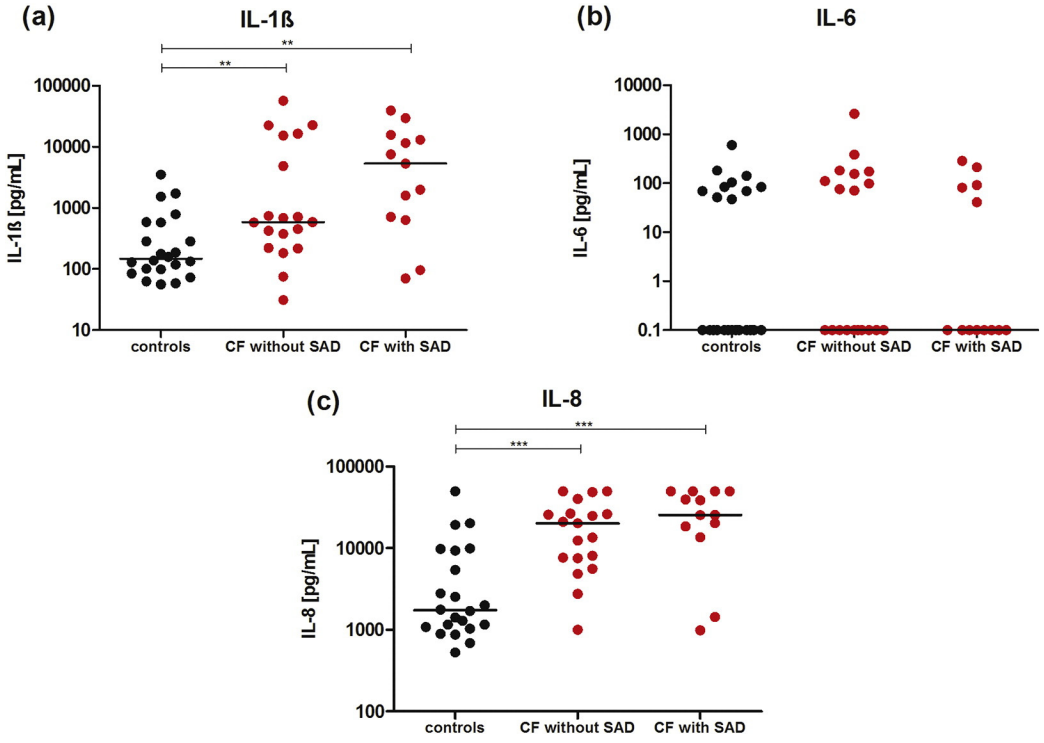


Fig. 3. Protein levels by CBA for selected cytokines/chemokines in sputum of CF patients and controls. Data for (a) IL-1ß, (b) IL-6, and (c) IL-8 are shown as median (range). The non-parametric Mann–Whitney test was used for comparisons between CF patients without and with SAD and healthy control subjects. Differences between CF groups and controls: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

functional lipid-raft-localized CFTR modulates NFκB-mediated inflammatory signaling and the innate immune response [22]. The effects of inflammatory cytokines, i.e. IL-1β, IL-6, and IL-8, may not be limited to actions on neutrophils. IL-8 may also act on airway smooth muscle and contribute to airway remodeling through promoting migration of smooth muscle cells within the airway wall [23].

Distinct patterns of airway remodeling and inflammatory biomarkers have emerged as important areas of investigation in an attempt to better understand and define ongoing inflammation and remodeling in CF. Airway inflammation in CF is associated with an abundant pro-inflammatory state, mainly driven by pro-inflammatory cytokines that result in neutrophilic invasion of sputum and tissue [3].

Controlling excessive neutrophilic airway inflammation is an important objective in the treatment of CF lung disease, especially in those patients with SAD. Therefore, selective inhibition of NFκB and the downstream IL-8 signaling pathways or treatment with CXCR2- or $LTB_4$-antagonists represents a promising target for future therapeutic strategies [24,25]. In the meantime, physicians caring for CF patients should be aware that the inflammatory response in mild CF patients is pronounced in patients with SAD. Our results show that this goes along with typical changes in lung function parameters, including a decrease in $MEF_{25}$ by means of spirometry and an elevated RV and RV/TLC by body plethysmography, which is more reliable and indicates airway trapping and small airway obstruction. Using the percent-predicted method of comparing an individual's lung function to that of a reference population has drawbacks. The use of percentiles or *Z* scores avoids this limitation. Further studies are needed to assess current lung function indicators with body plethysmographic data, sputum biomarkers, and LCI.

The study has several limitations: SAD was defined by spirometry and trapped air was detected by body plethysmography. Although, disease severity in CF is often stratified based on $FEV_1$, this parameter represents a poor prognostic disease marker for mild cystic fibrosis lung disease. New techniques like lung clearance index (LCI) may be more sensitive and may give better insight in SAD as recently described [26–28]. However, it is still unknown if involvement of SAD causes a more rapid decline of $FEV_1$ in the long run. Interestingly, data from CF piglets show no correlation of airflow limitations and inflammation in newborn pigs. The presence of air trapping, airflow obstruction, and airway size reduction in newborn piglets with cystic fibrosis before the onset of airway infection, inflammation, and mucus accumulation indicates that cystic fibrosis impacts airway development. These findings suggest that early airflow obstruction and air trapping in infants with cystic fibrosis might, in part, be caused by congenital airway abnormalities [29]. However, our study population was far beyond the newborn age. Hence, it is reasonable and furthermore a sign of advanced disease activity that our study patients with SAD, in contrast to patients without SAD, had significantly higher total cells and neutrophils in induced sputum. However, no significant differences on mediator levels could be demonstrated between both patient groups with the exception of IL6 on mRNA level. Taking into account recent data by Sagel et al., showing that

lung function decline was associated with increases in neutrophil counts, neutrophil elastase, and IL-1β [30], we may postulate that increased sputum neutrophil counts are associated with airflow limitations in the small airways. Changes in pro-inflammatory sputum biomarkers, like neutrophil counts are not solely related to changes in $FEV_1$ but may also help to describe a more advanced disease entity in the small airways. This study represents an important step in the validation of sputum inflammatory biomarkers as correlates of disease activity in the small airways. One drawback of the study is the inclusion of a broad age spectrum of patients. $FEV_1$ of 70% predicted in a seven-year-old patient provides much greater concern than a $FEV_1$ of 70% predicted in an adult patient with CF. In addition, more patients are needed in each group to perform multivariable analysis to determine if our panel of biomarkers can suggest involvement of the small airways.

In conclusion, our study demonstrated that patients with mild disease defined by lung function may be further endotyped according to their involvement of SAD. In CF patients with SAD, airway neutrophilic inflammation is more pronounced and is in part distinct from that seen in patients without SAD. Based on our results, sputum neutrophils, well known as implicated in disease pathogenesis and also in the development of bronchiectasis, appear to be the most informative individual biomarker to reflect disease activity in the small airways and represents a feasible treatment target in CF. A longitudinal study, which documents the change in FEV1, MEF25, LCI, sputum biomarkers, and imaging scores over time, might help to clarify the impact of new treatment modalities for the small airways in order to prevent progressive lung damage.

## Conflict of interest statement

None of the authors has a financial relationship with a commercial entity that has any interest in the subject of this manuscript.

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