ORIGINAL ARTICLE

Distinct patterns of inflammation in the airway lumen and bronchial mucosa of children with cystic fibrosis

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ABSTRACT

Background Studies in cystic fibrosis (CF) generally focus on inflammation present in the airway lumen. Little is known about inflammation occurring in the airway wall, the site ultimately destroyed in end-stage disease.

Objective To test the hypothesis that inflammatory patterns in the lumen do not reflect those in the airway wall of children with CF.

Methods Bronchoalveolar lavage (BAL) fluid and endobronchial biopsies were obtained from 46 children with CF and 16 disease-free controls. BAL cell differential was assessed using May-Gruenwald-stained cytospins. Area profile counts of bronchial tissue immunopositive inflammatory cells were determined.

Results BAL fluid from children with CF had a predominance of neutrophils compared with controls (median 810×10^3/ml vs 1×10^3/ml, $p<0.0001$). In contrast, subepithelial bronchial tissue from children with CF was characterised by a predominance of lymphocytes (median 961 vs 717 cells/mm^2, $p=0.014$), of which 82% were (CD3) T lymphocytes. In chest exacerbations, BAL fluid from children with CF had more inflammatory cells of all types compared with those with stable disease whereas, in biopsies, only the numbers of lymphocytes and macrophages, but not of neutrophils, were higher. A positive culture of *Pseudomonas aeruginosa* was associated with higher numbers of T lymphocytes in subepithelial bronchial tissue (median 1174 vs 714 cells/mm^2, $p=0.029$), but no changes were seen in BAL fluid. Cell counts in BAL fluid and biopsies were positively correlated with age but were unrelated to each other.

Conclusion The inflammatory response in the CF airway is compartmentalised. In contrast to the neutrophil-dominated inflammation present in the airway lumen, the bronchial mucosa is characterised by the recruitment and accumulation of lymphocytes.

Key messages

What is the key question?
- Do inflammatory patterns in the airway lumen of children with cystic fibrosis (CF) reflect those in the airway wall, the site ultimately destroyed in end-stage disease?

What is the bottom line?
- The inflammatory patterns and responses to infective stimuli in the airway lumen and the airway wall of children with CF are distinct, and thus bronchoalveolar lavage and endobronchial biopsy provide different but complementary information.

Why read on?
- This paper provides a detailed description of inflammatory cellular processes in the airways of subjects with CF in early disease stages.

INTRODUCTION

Pulmonary disease is the most significant cause of morbidity and mortality in cystic fibrosis (CF).[1] A hallmark of CF lung disease is chronic bacterial infection and the predominance of large numbers of neutrophils in the airway lumen. Neutrophils—which release an array of mediators, oxidants and proteases, including neutrophil elastase—are thus considered to play a major role in ensuing tissue damage and CF disease progression. In contrast to the large number of studies that have used bronchoalveolar lavage (BAL) to focus on the lumen, the CF airway wall has been underinvestigated. Indeed, little is known about the nature of inflammation in the airway wall, particularly that which occurs in the bronchial mucosa.

Studies in asthma have shown poor agreement between the patterns of inflammation in the airway lumen and that occurring in the bronchial mucosa,[2] suggesting that BAL and endobronchial biopsy measure dissimilar patterns of inflammation in these distinct compartments. In the few studies that have assessed airway wall inflammation in CF, the predominance of neutrophils seen in BAL fluid was not present in the bronchial wall.[3–5] Quantitative assessment of inflammatory cells in the bronchial mucosa has shown an accumulation of lymphocytes, especially at the distal level where intense tissue damage is observed. In contrast, neutrophils appeared to accumulate preferentially in the surface epithelium, suggesting migration of these cells towards the airway lumen.[4] As these studies have used end-stage CF lung tissue obtained at autopsy or transplantation, data on the pattern of inflammation in the bronchial mucosa in earlier stages of CF lung disease are lacking.

Airway disease is present early in life in CF, even in asymptomatic infants. Infection and inflammation can be detected in the BAL fluid of infants with CF even at a few weeks of age.[6] Structural airway wall changes, referred to as airway remodelling, also begin early in life in CF. CT scanning demonstrates the

presence of thickened airway walls, narrowed airway lumina, air trapping and bronchiectasis in young children and infants,[7–9] and lung function has been shown to be diminished in infants with CF.[10 11] However, the sequence of inflammation and remodelling events in the disease process is poorly understood.[12] Airway remodelling seems, at least in part, to be unrelated to airway lumen inflammation,[13 14] but its relationship to the inflammatory infiltrate in the bronchial mucosa has not been explored. Endobronchial studies during early stages of CF lung disease could assess to what extent—if at all—the patterns of inflammation in the airway lumen reflect airway wall changes, shed light on pathophysiological processes occurring in the airway wall and possibly allow assessment of longitudinal changes.

Based on the findings in asthma which show that differing yet complementary information is gleaned from BAL and endobronchial biopsy, we hypothesised that the CF airway inflammatory processes would be compartmentalised and that the luminal patterns of inflammation would not reflect changes in the bronchial mucosa. We tested this hypothesis using BAL fluid and endobronchial biopsies obtained from children with CF and disease-free controls.

METHODS

Subjects

Subjects were prospectively recruited between March 2003 and June 2007 from children with CF diagnosed using standard criteria[15] who were undergoing bronchoscopy for a clinical indication and control children who had been referred to a tertiary centre, but in whom no lower airway disease was found upon investigations that included clinically indicated flexible bronchoscopy. Specifically, inclusion criteria for control children were: (1) no prior history of lower airway disease; (2) no medication taken; (3) no macroscopic signs of inflammation seen on bronchoscopy (ie, no abnormal secretion, mucosal oedema or redness); (4) no viral detection or growth of pathogens in BAL fluid; and (5) BAL fluid with normal differential cell counts (ie, >80% macrophages).[16] One control was a child undergoing cardiac catheterisation where informed consent was given to perform bronchoscopy for research reasons. Exclusion criteria for both subject groups were bleeding tendency or treatment with anticoagulants and preterm birth before 36 weeks of gestation. Data from some of the children included in the present study have been previously reported.[13 17–20] The study was approved by the Royal Brompton Harefield and NHLI ethics committee. Informed consent and age-appropriate assent was obtained from parents and children respectively.

Flexible bronchoscopy

Bronchoscopy was performed as previously described under general anaesthesia.[18] BAL was performed using three aliquots of 1 ml/kg 0.9% saline at room temperature instilled separately into one or two lobes (usually the right middle lobe and the clinically most affected lobe), and the returns pooled. Up to five endobronchial biopsies (median 3, range 1–5) were taken from a standardised site (subsegmental bronchi of the right lower lobe) and processed into paraffin wax blocks as described previously.[18] In order to be included in the study, each child was a priori required to have at least one evaluable biopsy[18] with at least 0.1 mm^2 of subepithelial tissue.[21]

BAL fluid analysis

BAL fluid was processed as described previously.[13] Viable cells were counted and cell differentials were assessed on cytospin preparations using May-Gruenwald-Giemsa staining. Bacterial and fungal growth was assessed and viruses (respiratory syncytial virus, parainfluenza 1, 2 and 3, influenza A and B, adenovirus and cytomegalovirus) were sought using direct immunofluorescence and/or rapid viral tissue culture.

Immunohistochemistry

Endobronchial biopsies were immmunostained for neutrophils (neutrophil elastase), T lymphocytes (CD3) and B lymphocytes (CD20) lymphocytes, macrophages (CD68), eosinophils (EG2) and mast cells (tryptase). Area profile counts of immunopositive cells in subepithelial tissue were performed by one of two investigators (NR and LT) blinded to the study groups.[22]

Tissue morphometry

Morphometry was performed on 3 μm thick haematoxylin and eosin-stained biopsy sections as described previously.[13 19] Reticular basement membrane (RBM) thickness was measured on coded sections by taking the geometric mean of 40 measurements at 20 μm intervals.[19 23] The airway smooth muscle (ASM) volume fraction was measured, applying point and line intersection counting using previously recommended and validated methods of design-based stereology.[19]

Power calculation

Biopsy data from a study in atopic adults indicate that a sample size between 13 and 48 subjects is needed to detect at least one doubling difference in cell number per 0.1 mm^2 for a particular inflammatory cell type in a study using a parallel design such as ours, with α=0.05 and power of 0.80.[24]

Statistical analysis

Categorical data were examined using a χ^2 test. For normally distributed data, shown as mean (SD), between-group comparisons were performed with Student t tests and associations tested by Pearson correlation.[25] For non-normally distributed data, shown as median (range or IQR), between-group comparisons were performed with Mann–Whitney U-tests and associations tested by Spearman rank correlation. Multivariable linear regression models were used to adjust for possible confounders. Additional details are provided in the online supplement.

RESULTS

Patients

The study included 62 children of median age 7.3 years (range 0.2–16.8). Forty-six children had CF and underwent bronchoscopy for a clinical indication, and there were 16 controls without lower airway disease (table 1).

Neutrophilic inflammation in BAL fluid

Inflammatory cell counts in BAL fluid were available in 33 (72%) of the children with CF and in 10 (63%) controls, and cell differentials were available in 35 (76%) of the CF children and in 12 (75%) controls.

BAL fluid from children with CF contained more inflammatory cells and more of every cell type compared with controls (table 2). There was a predominance of neutrophils and macrophages, with moderate numbers of lymphocytes (figure 1A). In contrast, most of the inflammatory cells in control BAL fluid were macrophages. Mast cells were rarely seen in either CF or control BAL fluid (data not shown).

Biopsy analysis

A total of 181 biopsies were taken from the 62 children; 121 (67%) of these biopsies were considered evaluable, according to

Table 1 Subject characteristics (n=62)

	Cystic fibrosis (CF) (n=46)	Controls (n=16)	p Value
Sex, F:M	27:19	10:6	0.789
Age, years	7.8 (0.2–16.8)	6.3 (0.2–16.4)	0.334
FEV_1, % predicted*	53 (29–84)	98 (61–131)	**0.001**
FVC, % predicted*	74 (32–99)	93 (85–137)	**0.006**
CFTR genotype (n, %)	F508del/F508del (n=27, 59%); F508del/G542X (n=2, 4%); F508del/1717-1G>A (n=2, 4%); F508del/other (n=6, 13%); other or unknown (n=9, 20%).	ND	ND
Indication for bronchoscopy (n)	Microbiological surveillance at the time of chest exacerbation† (n=23) Routine microbiological surveillance at the time of new CF diagnosis; child well (n=8) or during chest exacerbation (n=7) Microbiological surveillance at the time of line or gastrostomy insertion or removal; child well (n=2) or during chest exacerbation (n=6)	Investigation of stridor (n=5; laryngo- and/or tracheomalacia found in 4 cases, double aortic arch found in one case) Investigation of recurrent croup (n=4; laryngo- and/or tracheomalacia found in 2 cases, large adenoids and tonsils found in 1 case, no pathology found in one case) Investigation of noisy breathing (n=2; no pathology found in either case, diagnosis of vocal cord dysfunction made in one case) Investigation of haemoptysis (n=2; no pathology found in either case) Investigation of recurrent cough (n=1; tracheomalacia found) Confirm diagnosis of vascular ring (n=1) Research bronchoscopy at the time of cardiac catheterisation (n=1)	ND
Pathogens in BAL fluid	Viruses: Parainfluenza virus 3 (n=2; 4%) Bacteria: *Pseudomonas aeruginosa* (n=11; 24%) Other (n=6; 13%)‡ Fungi: *Aspergillus* (n=13; 28%)§ *Candida albicans* (n=4; 9%)	No virus detection, no bacterial growth	ND

Values expressed as median (range) unless otherwise indicated.
*Data available for 29 children with CF and six control children.
†Chest exacerbation based on the decision to hospitalise and treat with intravenous antibiotics.[26]
‡*Staphylococcus aureus* (n=2), *Haemophilus influenzae* (n=1), *Mycobacterium chelonae* (n=2), *Stenotrophomonas maltophilia* (n=1).
§*Aspergillus fumigatus* (n=12), *Aspergillus niger* (n=1).
BAL, bronchoalveolar lavage; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; ND, not determined.

predefined criteria,[18] and had at least 0.1 mm^2 of subepithelial tissue.[21] Twenty-eight (45%) children had one evaluable biopsy, 16 (26%) had two, 13 (21%) had three, 3 (5%) had four and 2 (3%) had five. The median number of biopsies analysed per child was similar in the disease groups (2 (range 1–3) for CF and 1 (range 1–3) for controls, p=0.601), as was the median area of subepithelial tissue assessed (0.42 (range 0.16–0.76) mm^2 for CF and 0.15 (range 0.13–0.45) mm^2 for controls, p=0.152).

Lymphocytic inflammation in bronchial mucosa

There were more subepithelial inflammatory cells in CF tissue than in control tissue, with a similar cell distribution in both groups (table 2). CF subepithelial tissue was thus characterised by an infiltrate predominantly consisting of lymphocytes (median 961 cells/mm^2 vs 717 cells/mm^2, p=0.014) and macrophages, with few neutrophils, eosinophils or mast cells (figure 1B). The lymphocytic infiltrate consisted mainly of T lymphocytes both in CF tissue (82% T lymphocytes vs 18% B lymphocytes) and in control tissue (87% T lymphocytes vs 13% B lymphocytes).

Neutrophil, lymphocyte, macrophage and mast cell counts in BAL fluid were not correlated with counts for the same cell types in the biopsies. There was a weak relationship between eosinophil counts in BAL fluid and in subepithelial tissue within the CF group (r=0.43, p=0.019).

Relationship of airway inflammation with age, pulmonary function tests and CFTR genotype

Within the CF group, the number of BAL inflammatory cells (r=0.68, p<0.0001), neutrophils (r=0.64, p<0.0001), macrophages (r=0.44, p=0.011) and eosinophils (r=0.64, p<0.0001) correlated positively with age. In contrast, in the bronchial mucosa, only macrophage counts were positively related to age (r=0.32, p=0.042).

In the 29 children old enough to perform reliable spirometry, inflammatory cell counts in BAL fluid or biopsies were not related to any test value of pulmonary function (forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC) or FEV_1/FVC). There was no relationship between CFTR genotype and any inflammatory cell counts in BAL fluid or biopsies.

Different patterns of inflammation during chest exacerbations and phases of clinical stability in BAL fluid and biopsies

Bronchoscopic sampling had been performed during chest exacerbations—defined in terms of the decision to hospitalise and treat with intravenous antibiotics[26]—in 36 (78%) of the children with CF. The other 10 children underwent the procedure while well and did not require intravenous antibiotics either before or after the procedure. All inflammatory cell types in BAL fluid were higher in children investigated during chest exacerbations than in those who were well (table 3). These differences remained significant after adjustment for age for all cell types except for eosinophils. In contrast, in bronchial subepithelial tissue, only T lymphocytes and macrophages were significantly higher during chest exacerbations compared with those who were well. These differences remained significant after adjustment for age.

Table 2 Total and differential inflammatory cell counts in bronchoalveolar lavage (BAL) fluid and in subepithelial bronchial tissue obtained from children with cystic fibrosis (CF) (n=46) and controls (n=16)

	CF	Controls	p Value
BAL (cells $\times 10^3$ per ml)			
Total inflammatory cells	1382 (753–2951)	102 (78–190)	**<0.0001**
Neutrophils	810 (285–1230), 49%	1 (0–6), 1%	**<0.001**
Lymphocytes	37 (11–126), 2%	3 (1–5), 2%	**<0.001**
Macrophages	641 (329–1042), 48%	96 (73–168), 97%	**<0.0001**
Eosinophils	11 (1–41), 1%	0 (0–1), 0%	**0.008**
Biopsies (cells/mm^2)			
Total inflammatory cells	1773 (1256–2626)	1045 (760–1317)	**0.008**
Neutrophils	13 (4–26), 1%	0 (0–31), 0%	0.100
T lymphocytes	786 (648–1364), 54%	624 (439–788), 63%	**0.035**
B lymphocytes	125 (52–259), 9%	62 (28–179), 6%	0.100
Macrophages	487 (235–793), 33%	255 (102–446), 26%	**0.017**
Eosinophils	0 (0–0), 0%	5 (0–12), 0%	0.279
Mast cells	40 (14–78), 3%	52 (26–82), 5%	0.641

BAL cell counts and differentials available from 33 children with CF and 10 controls; biopsy cell counts available from 39–42 children with CF and from 13–16 controls, depending on cell type analysed; median (IQR) cell counts and percentage of total inflammatory cells shown.

Pathogenic organisms: distinct associations with luminal and mucosal inflammation

Pathogenic organisms were identified in 26 (56%) of the 46 children with CF (table 1). The most frequent pathogens were *Aspergillus* spp (n=13) and *Pseudomonas aeruginosa* (n=11). In one child both *Aspergillus* spp and *P aeruginosa* were identified.

The presence of *Aspergillus* spp was associated with higher numbers of BAL fluid inflammatory cells (median (IQR) 2898 (1377–6737) vs 1098 (670–2193) $\times 10^3$/ml, p=0.014) and neutrophils (median (IQR) 1190 (817–5473) vs 387 (199–990) $\times 10^3$/ml, p=0.010). However, after adjustment for age, this association was lost (p=0.251 and p=0.235, respectively). No differences were seen in biopsy tissue.

The presence of *P aeruginosa* was associated with higher numbers of bronchial mucosal inflammatory cells (median (IQR) 2579 (1813–2723) vs 1335 (1165–2084) cells/mm^2, p=0.019), but here lymphocytes predominated (median (IQR) 1606 (861–2087) vs 918 (720–1423) cells/mm^2, p=0.036) and these were of the T lymphocyte subset (median (IQR) 1174 (812–1936) vs 714 (575–1198) cells/mm^2, p=0.029). These differences remained significant after adjustment for age. In contrast, no differences were seen in BAL fluid.

Inflammatory cell counts in BAL fluid or biopsies were unrelated to the presence of other pathogens.

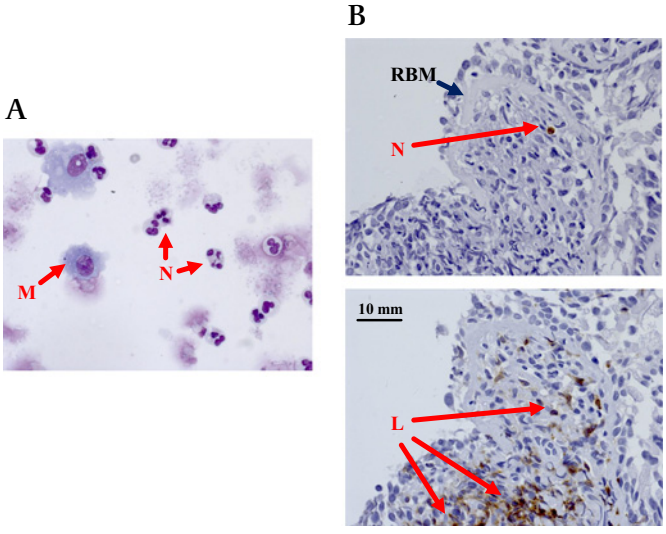

Figure 1 Representative high power view of a BAL sample obtained from a 10-year old CF child showing neutrophilic infiltration (A; May-Grünwald Giemsa, original×400) and of an endobronchial biopsy sample obtained from the same child showing dense lymphocytic infiltration with only few neutrophils in subepithelial tissue (B: top image: anti-NE staining for neutrophils; bottom image: anti-CD3 staining for T-lymphocytes, original×400). L, lymphocyte; M, macrophage; N, neutrophils; RBM, reticular basement membrane.

Relationship of airway inflammation and markers of airway remodelling

As we have previously reported in a smaller CF group including some of these cases,[13] RBM was thicker in children with CF than in controls (mean (SD) 4.8 (1.3) μm vs 3.9 (1.0) μm, p=0.008; figure E1 in online supplement). Within the CF group, RBM thickness was related to age (r=0.62, p<0.0001; figure E2 in online supplement), as shown in children without pulmonary disease.[27] After adjustment for age in a multivariable analysis, inflammatory cell counts in BAL fluid or biopsies were unrelated to RBM thickness (data not shown).

As also reported in a smaller group including some of these cases,[19] the volume fraction of ASM in bronchial tissue was larger in children with CF compared with controls (median (IQR) 0.11 (0.06–0.17) vs 0.06 (0.02–0.08), p=0.008; figure E4 in online supplement). Inflammatory cell counts in BAL fluid or biopsies were unrelated to the ASM volume fraction (data not shown).

DISCUSSION

Because of ease of access, most of the studies on inflammation and airway remodelling in CF have been performed on BAL fluid.[28 29] In contrast, the airway wall—the site of the destructive changes—has thus far been underinvestigated. This has been mainly due to difficulties in obtaining adequate material and also possibly to an assumption that the patterns of inflammation in the CF lumen provide a complete and reliable picture of those occurring in the airway wall per se. Recent advances in bronchoscopic techniques have allowed safe investigation of inflammatory and structural changes to the airway wall in CF, even in young children and infants.[18 30 31] In this study we used both BAL fluid and endobronchial biopsy to study inflammation in the airway lumen and the airway wall in children with CF as young as 2 months of age in order to

Table 3 Total and differential inflammatory cell counts in bronchoalveolar lavage (BAL) fluid and in subepithelial bronchial tissue obtained from children with cystic fibrosis (CF) during chest exacerbation[26] (n=36) or while well (n=10)

	Exacerbation	Well	p Value
BAL (cells $\times 10^3$ per ml)			
Total inflammatory cells	1914 (1098–3199)	512 (119–588)	**<0.001**
Neutrophils	970 (287–1584), 51%	222 (21–380), 56%	**0.004**
Lymphocytes	49 (18–150), 3%	10 (5–21), 3%	**0.019**
Macrophages	884 (433–1158), 46%	164 (94–319), 41%	**<0.001**
Eosinophils	17 (5–52), 1%	1 (0–3), 0%	**0.007***
Biopsies (cells/mm^2)			
Total inflammatory cells	1864 (1296–2634)	1263 (742–1549)	0.067
Neutrophils	14 (5–23), 1%	15 (0–31), 1%	0.964
T lymphocytes	912 (693–1411), 55%	563 (398–963), 59%	**0.034**
B lymphocytes	125 (66–197), 7%	102 (38–288), 11%	0.931
Macrophages	570 (316–881), 34%	187 (56–473), 20%	**0.005**
Eosinophils	0 (0–10), 0%	0 (0–4), 0%	0.215
Mast cells	45 (21–73), 3%	82 (15–178), 9%	0.379

BAL cell counts and differentials available from 27 children with CF during chest exacerbations and from six children with CF while well; biopsy cell counts available from 29–32 children with CF during chest exacerbations and from 8–10 children with CF while well, depending on cell type analysed; median (IQR) cell counts and percentage of total inflammatory cells shown.
*This significance was lost after adjustment for age (p=0.227).

compare the two. We demonstrate that inflammation is present in both compartments early in the course of the disease and that, as hypothesised, the patterns of inflammation in the lumen and bronchial mucosa are different. In contrast to the neutrophil-dominated inflammation in the airway lumen, CF is characterised by a lymphocytic inflammation of the bronchial mucosa. Furthermore, inflammatory associations with periods of exacerbation or CF-specific pathogens also differ at these two sites. These data demonstrate that BAL fluid and endobronchial biopsy provide different but complementary information, and both need to be sampled in order to be able to understand fully the pathophysiological processes in CF.

Our finding of the presence of large numbers of neutrophils in the BAL fluid is as expected and in agreement with multiple previous studies,[5 6 32] whereas the scarcity of neutrophils in the bronchial mucosa sampled by biopsy is novel. Our interpretation is that there is likely to be an intensive neutrophil recruitment from the bronchial vasculature followed by rapid migration through the bronchial wall with their accumulation in the airway lumen where they can be identified in high numbers in BAL fluid. Alternatively, neutrophils may enter the airway luminal compartment via tissue migration occurring more distally in the smaller membranous bronchioli. The data of Hubeau *et al*, however, who investigated the inflammatory infiltrate of the CF bronchial mucosa in transplantation tissue, would argue against this last speculation. The neutrophils identified in their study were particularly numerous at the segmental level compared with the lobar or distal level of the bronchial tree.[4]

Previous studies have described a lymphocytic infiltrate consisting mainly of T lymphocytes in the CF bronchial mucosa in autopsy or explant tissues recovered at end-stage disease.[3–5] Our findings in biopsy tissue of higher numbers of lymphocytes in the bronchial mucosa of children indicate clearly that their presence is not just a feature of end-stage disease but part of the ongoing inflammatory process of CF. Indeed, enhanced chemotaxis of CF compared with control lymphocytes towards interleukin 8, a predominant chemokine found in BAL fluid of subjects with CF,[33] has recently been shown in an in vitro setting.[34] The pathophysiological function of these infiltrating lymphocytes is unclear, but it could be speculated that T lymphocytes, the numbers of which we found to be higher during exacerbations and in the presence of *P aeruginosa*, may be involved in disease progression—for instance, through the induction of matrix metalloproteinase expression, known to be present in BAL fluid of subjects with CF and believed to be involved in airway tissue destruction.[35 36] In the study by Hubeau *et al* there were more T lymphocytes, particularly at the distal level of the bronchial tree where intense tissue damage was observed.[4] We are currently investigating further their role in early CF lung disease.[20]

Attention has recently turned to the role of alveolar macrophages in progression of CF lung disease.[37 38] Brennan *et al* reported higher absolute macrophage numbers but lower percentages in BAL fluid obtained from preschool children with CF with evidence of bacterial infection compared with uninfected children, suggesting that relatively fewer macrophages present during a pulmonary infection may have a diminished ability to phagocytise the increasing population of dying neutrophils.[38] Our findings of both higher macrophage numbers and percentages in the airway lumen and mucosa during CF exacerbations would argue against this hypothesis. The contribution of these cells to the disease process requires further investigation, but data obtained from CF fetal tissue suggest their possible involvement in the early onset of inflammation in infants with CF.[39]

We observed that chest exacerbations manifest differently in the bronchial lumen (higher numbers of all inflammatory cell types) and in the mucosa (higher numbers of lymphocytes and macrophages, but not of neutrophils). The higher numbers of submucosal lymphocytes during exacerbations are reminiscent of observations made in mild asthma exacerbations,[40] but the lack of higher numbers of submucosal neutrophils contrasts with findings reported in exacerbations of severe asthma[41] and chronic obstructive pulmonary disease,[42] suggesting that mechanisms of exacerbation are disease-specific. It could be speculated that the main source of neutrophil chemoattractants in the CF lung derives from the airway lumen (eg, soluble bacterial products, complement fragments or substances produced by airway luminal cellular components) or from the apical part of the airway epithelium[33 34] whereas, in other chronic airway diseases, neutrophil chemoattractants such as

CXCL8 (interleukin 8) or CXCL5 (ENA-78) originate from the submucosa.[41 42] We also found that different organisms had differential associations with luminal and mucosal inflammation. While the presence of *P aeruginosa* was associated with higher numbers of lymphocytes in the bronchial mucosa, no cell count differences between *Pseudomonas*-positive and *Pseudomonas*-negative patients were seen in BAL fluid, suggesting a role for submucosal lymphocytes in host defence against this pathogen as shown in animal models.[43 44] Chronic infection with *P aeruginosa* has clear detrimental effects on lung function and prognosis, perhaps underscoring the role of submucosal lymphocytic inflammation in disease progression. In contrast, we observed higher numbers of neutrophils in BAL fluid in the presence of *Aspergillus* spp, but no cell count differences between *Aspergillus*-positive and *Aspergillus*-negative patients were seen in the biopsies. However, the association in BAL fluid was lost after correction for age, suggesting that the presence of *Aspergillus* spp in BAL fluid is rather a marker of more severe inflammation and advanced or prolonged lung disease (ie, older age) than independently associated with disease progression, as recently proposed.[45] In our study we could not differentiate between colonisation and infection with pathogens; further work is needed to clarify their relationship with both luminal and mucosal inflammation.

Study limitations

We acknowledge that one reason for the lack of correlation of cells in BAL fluid with cells in biopsies could be technical, as endobronchial biopsy and BAL sample different areas of the airways. Endobronchial biopsy samples come from relatively proximal large airways and the airway mucosa of bronchial bifurcations (carinae) and they do not represent the full airway wall thickness. Our data may therefore not be representative of the entirety of the conducting airways, and we may have missed changes occurring in subsegmental airways and therefore associations with, for example, spirometry, even though spirometry is probably too insensitive to detect subtle changes.[46] Endobronchial biopsies are further limited in size and there is considerable variability between samples of a given individual, so multiple biopsies per subject should ideally be analysed.[47 48] Endobronchial biopsy in children is challenging,[18 31] and we were only able to analyse 1–2 biopsies of adequate morphology per child. Although our study was powered sufficiently to detect between-group differences,[24] some relationships within individuals may thus have been missed due to inadequate sampling. The findings of our study need to be interpreted in light of these limitations. However, the strength of our data lies in the relatively large number of biopsies studied and in the inclusion of disease-free controls.

Conclusions

Our data show that inflammation is present in both the airway lumen and the bronchial mucosa but that, in support of our hypothesis, the patterns of inflammation and the responses to infective stimuli observed in BAL fluid and biopsies obtained from children with CF are dissimilar. In contrast to the neutrophil-dominated inflammation present in the airway lumen, CF is characterised by a lymphocytic infiltration and accumulation of the bronchial mucosa, the function of which merits further investigation. BAL and endobronchial biopsy provide different but complementary information, underscoring the need to apply both techniques in the study of the pathophysiological processes in the CF airway. Considering that progressive airway wall changes ultimately lead to bronchiectasis, we suggest that further research should focus on understanding the pathogenetic mechanisms at this tissue site.

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethics approval was provided by Royal Brompton Harefield and NHLI ethics committee.

Contributors Conception and design of the study: NR, TNH, EWFWA, PKJ, AB, JCD. Acquisition of data: NR, LT, TNH, HT, JZ, Y-SQ, AB, JCD. Analysis and interpretation of data: NR, OF, EWFWA, PKJ, AB, JCD. Drafting and revising: NR, OF, PKJ, AB, JCD. Important intellectual content: NR, TNH, EWFWA, PKJ, AB, JCD. Final approval: all authors.

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Journal club

Role of kinase suppressor of Ras-1 in *Pseudomonas aeruginosa* infections

Respiratory infection with *Pseudomonas aeruginosa* can have serious implications, particularly on a background of immunodeficiency, cystic fibrosis and mechanical ventilation. In this study, by conducting a series of experiments on mice, the authors identified the key role of the kinase suppressor of Ras-1 (Ksr1), an enzymatic protein, in the innate host response to *P aeruginosa* infection.

Ksr1 deficiency impairs the bactericidal activity of alveolar macrophages and, as a consequence, Ksr1-deficient mice were found to die of sepsis from failed clearance of *P aeruginosa*. The bactericidal activity of alveolar macrophages and neutrophils is mediated by the formation and release of nitric oxide (NO) and peroxynitrite, which is triggered by Ksr1. This occurs through a previously unidentified pathway where Ksr1 functions as a unique scaffold and mediates the interaction between inducible NO synthase (iNOS) and heat shock protein 90, thereby activating iNOS and releasing NO, which kills the bacteria.

The authors concluded that this study identifies a unique role of Ksr1 in bacterial infection and they have shown a link between Ksr1 and the regulation of bacterial pneumonia and sepsis.

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Distinct pattern of inflammation in bronchoalveolar lavage and bronchial mucosa of children with cystic fibrosis

Online Repository

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METHODS

Subjects - CF children

All CF children undergoing flexible bronchoscopy for a clinical reason at the Royal Brompton Hospital between March 2003 and June 2007 (n= 183) were considered for participation in the study. One-hundred and seven CF children were recruited. Sufficient biopsy material (see inclusion criteria below) was available in 46 of them. These children had following CFFTR genotypes: F508del/F508del (n=27, 59%); F508del/G542X (n=2, 4%); F508del/1717-1G>A (n=2; 4%); F508del/other (n=6, 13%); other or unknown (n=9, 20%). CF diagnoses had been made clinically, as CF newborn screening had not been implemented at the time of this study.

Flexible bronchoscopy

Depending on the size of the child, different bronchoscopes were used: BFXP40 (2.8 mm external diameter), BF-3C20 or 3C40 (3.6 mm external diameter), or BF-MP60 (videobronchoscope, 4.0 mm external diameter), or BF-P20D (4.9 mm external diameter), all from Olympus (Tokyo, Japan). Up to 5 endobronchial biopsies were taken under direct vision from a standardized site (i.e. sub-segmental bronchi of the right lower lobe). Small reusable forceps (FB-56D, oval cup with rat tooth jaw; KeyMEd; Southend-on-Sea, Essex, UK) were used with the 2.8-mm or 3.6-mm bronchoscope (both with a 1.2-mm working channel). Large reusable forceps (FB-19-C1, oval cup standard; KeyMed) or single use forceps (FB-231D, oval cup standard; KeyMed) were used with the 4.0-mm or 4.9-mm bronchoscope (working channel 2.0 vs. 2.2 mm, respectively).

Bronchoalveolar lavage (BAL)

BAL was performed for clinical reasons in all children, and was primarily used for microbiological assessment. Therefore, in some cases, there was not enough material left for cell counts.

Biopsy processing and staining

Biopsies were fixed in 10% formal saline solution overnight and processed into paraffin blocks. One 3 μm section was stained with haematoxylin and eosin and categorized as ‘evaluable` or ‘non-evaluable’. To be categorized as “evaluable”, a biopsy had to fulfill following criteria: (i) presence of epithelium, reticular basement membrane (RBM) and

subepithelial tissue; (ii) good orientation; (iii) minimal crush, edema or blood within the biopsy (E1). Biopsies with 'evaluable' sections were then cut further and up to ten 3 μm sections were then taken at 50 μm intervals and stained with monoclonal mouse anti-human neutrophil elastase (NE)(M0752, DAKO, Glostrup, Denmark) for neutrophils, polyclonal rabbit anti-human CD3 (A0452, DAKO, Glostrup, Denmark) for T-lymphocytes, monoclonal mouse anti-human CD20cy (M0755, DAKO, Glostrup, Denmark) for B-lymphocytes, monoclonal mouse anti-human CD68 (M0876, DAKO, Glostrup, Denmark) for macrophages, monoclonal mouse anti-human eosinophilic cationic protein (EG2)(Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) for eosinophils and monoclonal anti-tryptase (M7052, DAKO, Glostrup, Denmark) for mast cells (E2-E4). Neutrophils, T- and B-lymphocytes and macrophages were identified using the DAKO Autostainer streptavidin method® (DAKO, Glostrup, Denmark) after heat-mediated antigen retrieval by pressure cooking in 0.01M citrate buffer (except for neutrophils, for which no pre-treatment was needed). Eosinophils and mast cells were identified using the EnVision-alkaline phosphatase (EV-AP) technique (DAKO, Glostrup, Denmark), as previously described (E5). Some biopsies did not yield enough sections to perform all stains.

A subset of the biopsies (n=30) from CF children was also stained with monoclonal mouse anti-human CD83 (VP-C368, Vector, Burlingame, Ca, USA) for mature dendritic cells (DCs), as previously described (E6). However, there were only very few positive cells in these samples (1-2 positive cells in only 3/30 biopsy samples), and therefore this stain was not performed for the rest of the biopsy samples.

Quantification of inflammatory cells on biopsies

Sections were coded and counted by two blinded observers (NR and LT). Areas of subepithelial tissue, excluding areas with mucus-secreting glands, bronchial smooth muscle and large vessels, were assessed using an Apple Macintosh computer and Image 1.5 software (Apple Computer, Cupertino, CA). To be included in the study, we required *a priori* that each child had at least one biopsy with at least 0.1 mm^2 of subepithelial tissue (E7).

Using a light microscope (Dialux 20, Leitz, Wetzlar, Germany) at x400 magnification, area profile counts were used to count inflammatory cells in the subepithelial tissue of each biopsy specimen. The data were expressed as the number of cut cell profiles with a nucleus visible (i.e., positive cells) per square millimeter of the subepithelium, the mean of all evaluable biopsy specimens representing the value for that subject.

Repeatability and variability

Intra-observer repeatability and within-observer, within-biopsy and between-biopsy variability were determined (E8). The mean intra-observer repeatability, expressed as coefficient of variation (C%V) for cell count measurements on four occasions ranged from 17.7% (T-lymphocytes) to 23.9% (B-lymphocytes, Table E1).

Table E1. Repeatability and variability of cell count measurements, expressed as percent coefficient of variation (CV).

	Neutrophils	T-lymphocytes	B-lymphocytes	Macrophages	Mast cells	Eosinophils
Intra-observer repeatability	13.4	7.7	23.9	14.9	9.1	8.1
Within-biopsy variability	21.9	9.3	33.9	13.2	12.9	n.a.
Between-biopsy variability	130.2	64.2	51.5	63.8	76.9	173.2

Definition of abbreviation: n.a. = not assessed

Within a single biopsy, the between-section CV for four sections ranged from 9.3% (T-lymphocytes) to 33.9% (B-lymphocytes). Between-biopsy CV ranged from 51.5% (B-lymphocytes) to 173.2% (eosinophils). Overall inter-observer agreement of the two blinded observers (NR and LT) for cell counts was good (ICC=0.87) and ranged from 0.61 (neutrophils) to 0.95 (mast cells). These results are similar to those previously published (E9).

Reticular basement membrane (RBM) thickness

Reticular basement membrane (RBM) thickness was measured on 3 μm thick haematoxylin and eosin-stained coded sections as previously described (E10, E11). One section of each biopsy was selected which showed identifiable epithelium and submucosal with at least 800 μm of RBM. RBM thickness was measured by a blinded observer (NR) using light microscopy and computer-aided image analysis (NIH Image 1.55; National Institutes of Health, Bethesda, Maryland, USA) by taking the geometric mean of 40 measurements at 20 μm intervals. The mean intra-observer repeatability as coefficient of variation (CV) for RBM thickness measurements on four occasions was 5.2%. Within a single biopsy, the between-

section CV for seven sections was 18.9%. The mean [SD] between-biopsy CV obtained from 10 patients in whom RBM thickness was measured in 3 biopsies was 15.8 [6.6]%.

Airway smooth muscle (ASM) mass

Airway smooth muscle (ASM) mass was assessed on 3 μm thick haematoxylin and eosin stained sections using equations from design-based stereology (E12, E13), as described previously (E14). The ASM volume fraction was measured using point and line intersection counting. Briefly, the numbers of points overlying ASM and other subepithelial tissue and the number of lines intersecting the apical surface of RBM by light microscopy were recorded using a x10 lens and a M168 counting grid (x390 total magnification, Figure E3).

Stereological data were calculated from point and line intersection counts as follows:

(1) volume fraction of ASM indexed to volume of subepithelial tissue: Vv (sm/subepithelium) = (Σ points on ASM) / (Σ points on subepithelial tissue)

(2) volume fraction of ASM indexed to surface area of RBM: V/S (sm/rbm) = (Σ points on ASM x l(p)) / (2 x Σ line intersections with RBM); where l(p) denotes length per point (μm)

Statistical analysis

Data were analyzed on a ‘per individual’ as opposed to ‘per biopsy’ basis, e.g. the sum of the measurements obtained from all biopsies of a given subject was taken as value for this subject. SPSS v15 (SPSS Inc, Chicago, IL, USA) and Stata IC 11.0 for Windows (StataCorp, College Station, TX, USA) were used for statistical analysis.

Linear regression

Having found a positive association of inflammatory cell counts with age within the CF group, we performed multivariable regression analyses to adjust group differences for age for all subsequent analyses done within the CF group. Multivariable models were fitted with parameters significantly associated with outcomes (numbers of inflammatory cells) in univariable models (i.e. presence of chest exacerbation, presence of *Aspergillus sp.* and presence of *Pseudomonas aeruginosa* in BAL). We tested whether these parameters remained significantly associated with outcomes after a backward stepwise exclusion strategy of dropping the explanatory variable with the highest p-value until only significant associations

were left in the final model. A p-value <0.05 was considered significant. For linear regression analyses, non-normally distributed cell counts were transformed to normalize their distribution (log-transformation for total cell counts, neutrophils and macrophages in BAL and total cell counts in biopsies; square-root transformation for lymphocytes and eosinophils in BAL and for neutrophils, macrophages, lymphocytes and eosinophils in biopsies).

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LEGENDS TO THE SUPPLEMENTAL FIGURES

Figure E1. Reticular basement membrane (RBM) thickness in biopsies obtained from cystic fibrosis (CF) children (n=46) and controls (n=16). RBM was significantly thicker in the CF group. Horizontal bars represent means.

Figure E2. Panel A: Relationship between reticular basement membrane (RBM) thickness in biopsies obtained from cystic fibrosis (CF) children (n=46) and age. Panel B: Relationship between reticular basement membrane (RBM) thickness in biopsies obtained from control children (n=16) and age.

Figure E3. Representative low power view (x200) of an endobronchial biopsy section stained with haematoxylin and eosin with superimposition of a M168 counting grid, allowing the measurement of ASM volume fraction

Figure E4. Airway smooth muscle (ASM) content in endobronchial biopsies from children with cystic fibrosis (CF, n=46) compared to control children (n=16). *Definition of abbreviations*: Vv (sm/subepithelium) = volume fraction of ASM indexed to volume of airway subepithelial tissue. Horizontal bars represent medians.

215**Figure E1**

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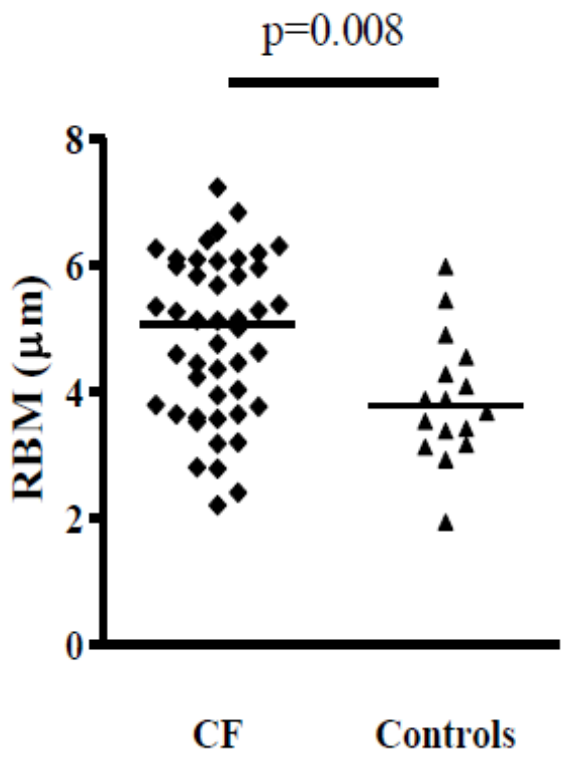

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219**Figure E2**

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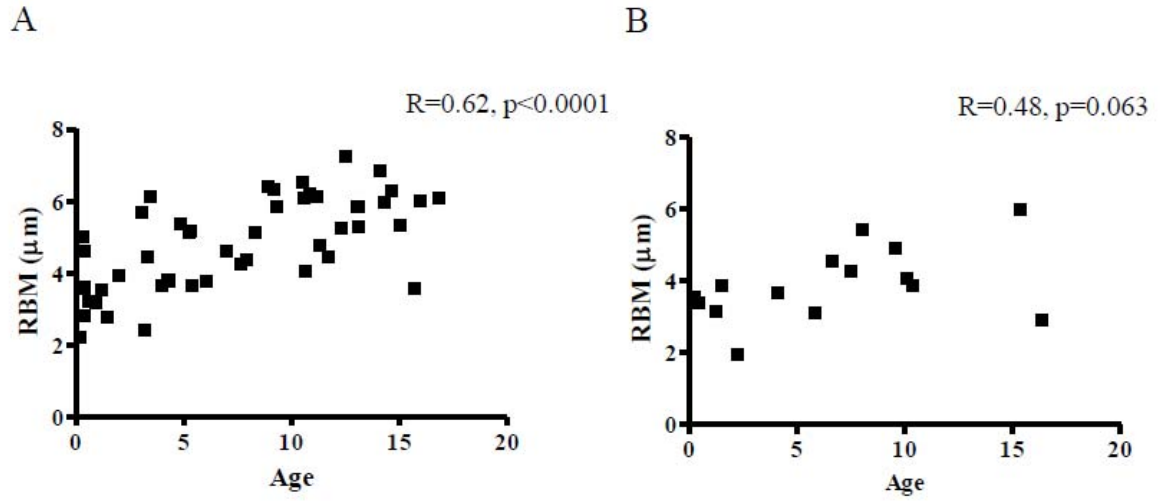

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222**Figure E3**

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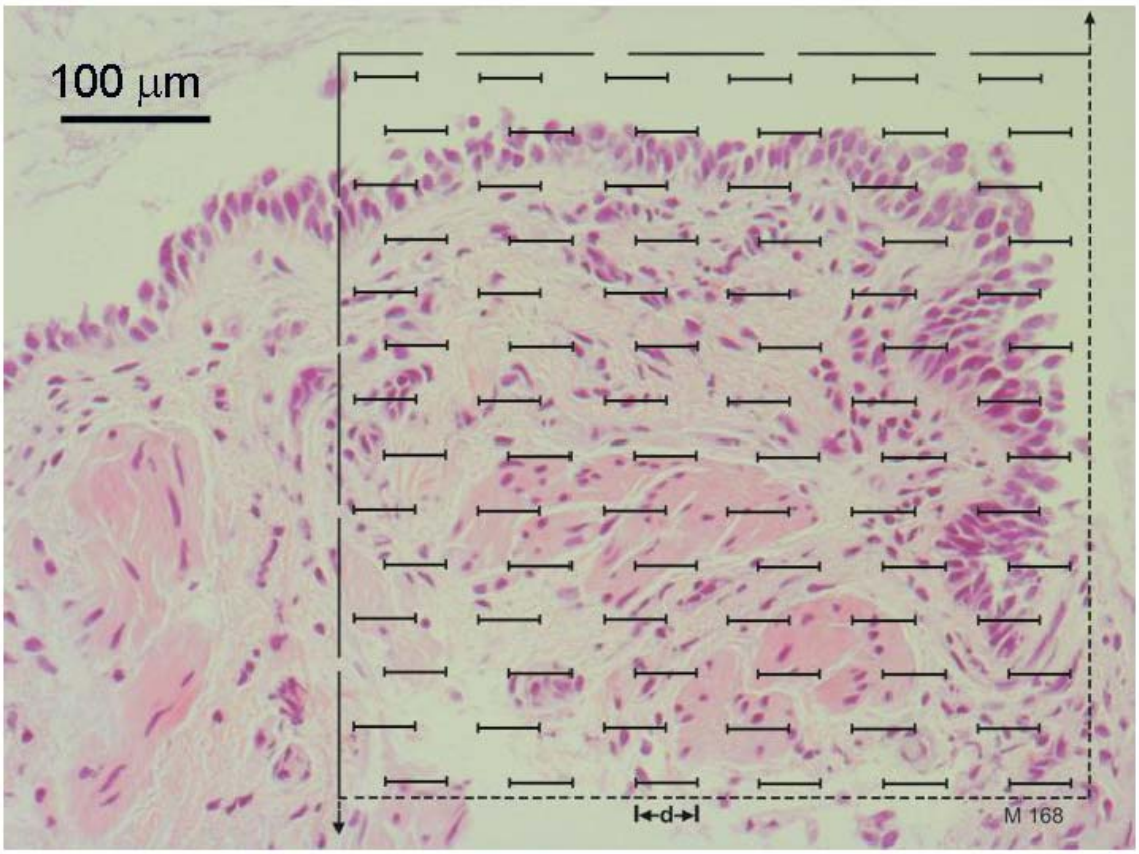

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225**Figure E4**

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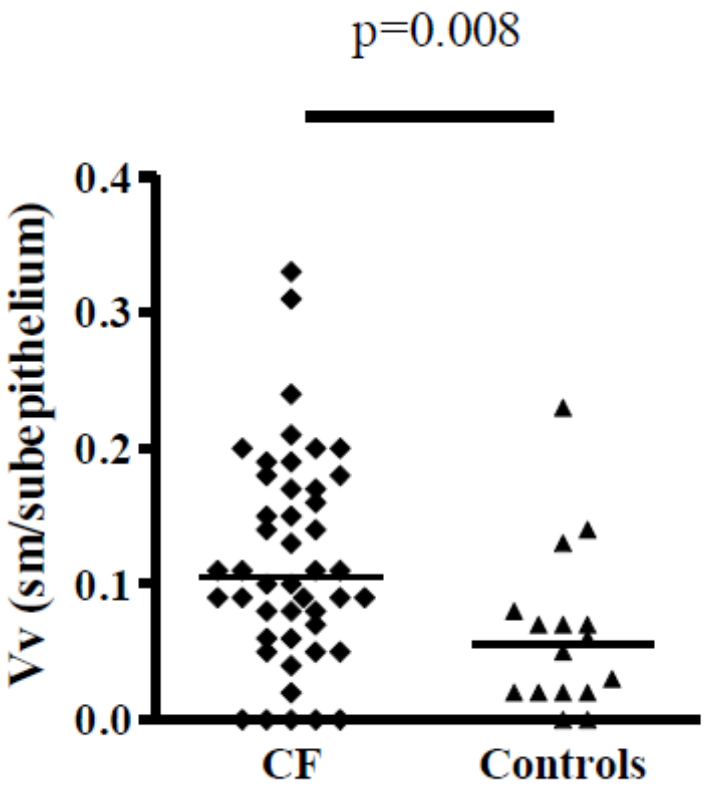

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