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**High concentrations of pepsin in bronchoalveolar lavage from children
with cystic fibrosis are associated with high interleukin-8**

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ABSTRACT

Background: Gastro-oesophageal reflux (GOR) is common in children with cystic fibrosis (CF) and is thought to be associated with pulmonary aspiration of gastric contents. Recently the measurement of pepsin in bronchoalveolar lavage (BAL) has been suggested to be a reliable indicator of aspiration. We assessed the prevalence of pulmonary aspiration in a group of children with CF and investigated its association with lung inflammation.

Methods: This was a cross-sectional case-control study. BAL was collected from individuals with CF (n=31) and healthy controls (n=7). Interleukin-8 (IL-8), pepsin, neutrophil numbers and neutrophil elastase (NE) activity levels were measured in all samples. Clinical, microbiological and lung function data were collected from medical notes.

Results: BAL pepsin concentration was higher in the CF group (mean 24.4 [SD 27.4] ng/ml) compared to controls (4.3 [4] ng/ml, $p=0.03$). Those with CF who had elevated pepsin concentrations had higher levels of BAL IL-8 (3.7 [2.7] ng/ml) compared to those with a concentration comparable to controls (1.4 [0.9] ng/ml, $p=0.004$). Within the CF group, we observed a moderate positive correlation between pepsin concentration and IL-8 in BAL ($r=0.48$, $p=0.04$). There was no association between BAL pepsin concentrations and age, sex, BMI z score, FEV₁ or *P. aeruginosa* colonisation status.

Conclusions: Many children with CF have elevated BAL pepsin levels compared to normal controls. Elevated pepsin levels were associated with higher IL-8 concentrations in BAL. These data suggest that aspiration of gastric contents occurs in a subset of patients with CF and is associated with more pronounced lung inflammation.

BACKGROUND

The symptoms of gastro-oesophageal reflux (GOR) are very common in children with cystic fibrosis¹. Studies using oesophageal manometry and pH probes have demonstrated high levels of GOR in both children and adults with cystic fibrosis. Scott et al found GOR in 20% of those less than 5 years of age while Ledson et al, showed a prevalence of 80% in adult patients, some of whom showed tracheal acidification in addition, suggesting micro-aspiration¹⁻³. According to Blondeau et al, GOR is common in adults with CF, and associated with increased cough and poorer lung function⁴. Several patients in that study had bile acids in their BAL, suggesting a link with aspiration. A study by Button et al found that GOR was present in 90% of severely-affected adult patients with CF pre-transplant and 84% post-transplant⁵.

Diagnosis and treatment of GOR is a common component of care at most CF centres. Many patients will report symptom improvement with gastric acid suppressive therapy. However, treatment with acid blockade will not prevent non-acid reflux or its aspiration⁶. The detection and treatment of pulmonary aspiration is fraught with difficulty. The diagnosis is often made with a combination of clinical, laboratory and radiological tests. However, the overall sensitivity and specificity of these tests alone or in combination remains poor. An ideal test for aspiration would involve the detection of a marker within the lungs that clearly should not be present within the tracheobronchial tree under normal conditions. The marker ought to be easily recovered and reliably detectable for a period

of time after acute aspiration. Recently, several investigators have focused on pepsin, a digestive protease that degrades food proteins into peptides. It is expressed as a pro-form zymogen, pepsinogen, which is released by chief cells in the stomach and subsequently activated by hydrochloric acid (HCl) cleaving it to the active form, pepsin. A link between pulmonary aspiration and the detection of pepsin in tracheal aspirates or BAL has been demonstrated in human and animal studies⁷⁻¹⁶. Farrell et al, recently employed the pepsin ELISA used in this study to look specifically at aspiration of gastric contents in children without CF¹⁰.

Given the increased prevalence of gastro-esophageal reflux in children with CF¹⁻⁴, and the link between aspiration of gastric contents and lung inflammation¹⁷, we sought to determine whether pulmonary aspiration was occurring in children with CF, and if so, whether this was associated with more marked pulmonary inflammation.

METHODS

Patient selection

We studied a consecutive group of children with CF referred for bronchoscopy during the period July 2005 – July 2007 who satisfied the selection criteria and provided written informed consent. There was a range of different indications for these procedures; For routine microbiological surveillance in non sputum producers, to confirm or follow up a growth of *P. aeruginosa* in a throat swab, to investigate ongoing respiratory symptoms out of context with culture results, and in some cases, opportunistically at the time of other elective procedures in these patients. Inclusion criteria were as follows: Age 0-17 years, an abnormal sweat test and the presence of at least one CF mutation. Exclusion criteria were as follows: Presence of an acute CF exacerbation, a diagnosis of allergic bronchopulmonary aspergillosis, treatment with systemic steroids and a clinical diagnosis of asthma. Non-CF healthy control samples were obtained from children without lung disease undergoing elective non-pulmonary surgical procedures. Inclusion criteria were as follows: age 0-17 years. Exclusion criteria were as follows: Any history of recurrent respiratory symptoms, respiratory symptoms on the day of surgery or a diagnosis of asthma. Full informed parental consent was obtained for all procedures and ethical approval was obtained from the institutional review board of the Adelaide and Meath Hospital incorporating the National Children's Hospital, Dublin.

Bronchoalveolar lavage fluid

Bronchoscopy was performed via a laryngeal mask airway to avoid contamination from the upper airway and aspiration of gastric contents during the procedure. Bronchoalveolar

lavage was performed in the lingula and right middle lobe by instilling 1 ml/kg of sterile normal saline per lobe. Return was typically in the region of 40%. Specimens from right and left lungs were pooled. BAL fluid was centrifuged at 2000 x g for 10 min and the supernatant aliquoted and stored at -80°C.

Pepsin ELISA

Pepsin concentrations were measured in clinical samples by an 'in-house' ELISA assay with anti-porcine pepsin antibodies developed at the Queens University Belfast, as previously described¹⁰ (See appendix 1).

IL-8 ELISA

IL-8 was measured by ELISA using 1:125 mouse anti-IL-8 as primary antibody and 1:2500 biotinylated goat anti-IL-8 as detection antibody (all R&D systems, Abingdon, Oxon, UK).

Study design and sample size estimation

This was a cross-sectional case-control study. All relevant demographic, clinical, and laboratory data were collected from the patients medical notes and recorded in a dedicated case record form. There were limited data to inform sample size calculation, however estimates were calculated using the Satterthwaite t-test of equal means (unequal variances, unequal groups). Presuming a minimum average pepsin concentration of 24 ng/ml in cases (based on pepsin values in children with aspiration)¹⁰ and a maximum average pepsin concentration of <5 ng/ml in control subjects, with a ratio of cases to

controls of 5:1, and at two-tailed $p < 0.05$ (power > 85%), a minimum of 27 cases and 5 controls were required.

Statistical analyses

Descriptive statistics and percentile methods were used. The normality of the distribution of continuous variables was assessed by the Kolmogorov-Smirnov test. The groups and sub-groups were compared by two-tailed independent sample Student's t-test when normally distributed, or alternatively, by the non-parametric Mann-Whitney test, as appropriate. The associations between different variables among children with CF were evaluated by univariate analyses. Pearson's correlation coefficient was used for establishing correlations between normally distributed continuous variables, or, alternatively, the Spearman rho coefficient was used. Continuous data are expressed as mean and [standard deviation] or SD, unless otherwise specified. The statistical significance of all tests was assumed at two-tailed $p < 0.05$. All analyses were performed with GraphPad Prism software.

RESULTS

Study patients

Overall, 31 cases with CF and 15 controls were identified as eligible participants (see Table 1). After identification, only 8 controls did not provide written informed consent and did not participate and complete the study. Finally, out of all 46 identified subjects, a total of 38 subjects were recruited and analysed (n=31 cases, average age 10.4 years, range 0.4-17.7 years; n=7 controls, average age 8.3 years, range 3.7-15.1 years).

Clinical characteristics

There was no significant difference in age or gender distribution, *P. aeruginosa* colonisation status or previous investigations for GOR between the two groups of individuals with CF (CF with high versus CF with low BAL pepsin concentration, **Figure 2**).

BAL pepsin is elevated in individuals with CF

Mean pepsin levels in the CF group (24.4 [27.4] ng/ml) were higher than those in the control group (4.3 [4.0] ng/ml, $p=0.03$, **Figure 1**). It should be noted that some pepsin was detected in most samples, even controls. A large proportion (12 out of 31) of the CF group had pepsin levels within the same range as the control group. As a low concentration of pepsin was detectable in BAL from controls, the pepsin concentration in

those with CF were considered ‘high’ if it was $\geq 95^{\text{th}}$ percentile for controls (10.4ng/ml), and ‘low’ if they were below this value.

BAL pepsin correlates with IL-8 in individuals with CF

We sought to investigate whether this increase in pepsin values was associated with increased evidence of inflammation. BAL IL-8 levels were significantly higher in those with high pepsin levels (3.7 [2.7] ng/ml) compared to those with low pepsin (1.4 [0.9] ng/ml, $p=0.004$, **Figure 2**). Furthermore, there was a positive correlation between BAL pepsin and BAL IL-8 in the group with CF ($r=0.48$, $n=31$, **Figure 3**). Increased BAL neutrophil counts were observed in the CF group with high BAL pepsin concentrations compared to those with low BAL pepsin concentrations ($5.5 [6.9] \text{ v } 2.6 [3.8] \times 10^6$ cells/ml) although the difference was not statistically significant ($p_{\text{t-test}}=0.15$).

DISCUSSION

In this study of children with CF undergoing bronchoscopy as part of their clinical care, we found that in more than half, BAL pepsin concentrations were elevated in keeping with pulmonary aspiration. Moreover, those with higher BAL pepsin concentrations also had higher IL8 concentrations suggesting that ongoing pulmonary aspiration is contributing to airways inflammation

The diagnosis of pulmonary aspiration remains a challenge. No single test to date has been demonstrated to be 100% sensitive or specific, and the diagnosis is often based on clinical suspicion with some supporting evidence from radiological, laboratory and physiological tests. Up to now, the most widely used method for detection of pulmonary aspiration has been microscopic analysis of secretions for the presence of lipid-laden macrophages. An index comparing the proportion of lipid laden to normal macrophages must be calculated and used for comparison – the lipid laden macrophage index (LLMI). This test is labour intensive, operator dependent and has poor specificity and variable sensitivity^{18;19}. No clear reference standards exist for this method. The technique has been evaluated in children with CF. Kazachkov et al found elevated LLMI in children both with CF, and with other chronic pulmonary conditions, but no normal controls were assessed. The LLMI was higher in the CF group, but this did not correlate with inflammatory markers in BAL or symptoms suggestive of GOR in either group²⁰.

In situations where aspiration of gastric refluxate into the lungs is strongly suspected, the definitive treatment usually involves surgical fundoplication. However, surgical procedures in CF, especially those which may alter physiological function, are not undertaken lightly and robust evidence for their need should be obtained. The measurement of pepsin levels in BAL has the potential to provide such confirmatory documentation. Pepsin assays have the potential to significantly improve the sensitivity and specificity of the current diagnostic approach to aspiration in the clinical setting. The pepsin assay used in this study was specifically developed and validated for detection of pulmonary aspiration in children¹⁰.

The fact that pepsin is produced in the GI tract and not the lung fulfils one of the most important requirements of a reliable marker of aspiration. However a study in healthy young adults has shown that a significant portion of patients aspirate nasopharyngeal secretions during sleep²¹. In patients with GOR, where upper oesophageal sphincter tone drops during deep sleep, the potential exists for some micro-aspiration of gastric contents²². So the presence *per se* of pepsin, at any level, in the BAL may not necessarily always be an abnormal finding. In this context, the detection of low levels of pepsin in the BAL of some of our control patients is perhaps not surprising. Several of the individuals with CF have similarly low levels of pepsin, however many more had levels that were significantly elevated above control values, perhaps indicating more significant degrees of aspiration. A clear segregation between low and high pepsin can be observed using the 95th percentile cut off (Figure 1). There are no published data for the concentration of pepsin in the BAL that may be seen in the absence of respiratory

symptoms or pulmonary inflammation, so deciding which levels are potentially physiological and which are clearly pathological presents a challenge. Individuals in the control group were completely asymptomatic from a respiratory perspective at the time of bronchoscopy, and had normal differential cell counts and negligible levels of inflammatory cytokines (data not shown) in BAL. Therefore we feel confident that the pepsin levels measured in this group are unlikely to be clinically significant and represent a true normal range. The presence of low levels of pepsin in otherwise healthy children is surprising, but may reflect microaspiration during sleep as has been shown in adults. Little is known about how long pepsin may be detectable in the lungs after aspiration of gastric contents.

The finding of elevated levels of IL-8 among the group with the higher pepsin levels, and the correlation between levels of IL-8 and pepsin in BAL suggests that high pepsin levels are associated with more severe lower airway inflammation. Similar findings have been observed in lung transplant recipients^{16;23}. This is the first time that a link has been demonstrated between a biological marker of aspiration and inflammation in relatively stable individuals with CF (mean FEV₁= 66%). This association is strengthened by the fact that both measurements are derived from the same sample from any given patient, minimising the effect of even small variations in BAL concentration between patients. Our finding of the link between pulmonary aspiration and inflammation in CF is potentially an extremely important discovery for individuals with CF. Indeed the relevance is amplified when one considers that many of the children in the study had no

symptoms of GOR. Thus, it seems likely that silent aspiration of gastric contents may be a more common problem than most CF clinicians would anticipate.

Contrary to what one might expect, we found no increase in pepsin levels in BAL with age, nor did we see an association between pepsin levels in BAL and lung function. This may be the result of our small numbers, and the cross sectional nature of this study. It is likely that the degree of aspiration in patients varies over time²¹, so a longitudinal study may be better equipped to address these associations.

This study demonstrates for the first time that aspiration of gastric contents occurs in a subset of clinically stable patients with CF and is associated with more pronounced lung inflammation. Further longitudinal study is required to determine the long-term clinical significance of this observation.

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Figures and Tables

Table 1: Baseline characteristics and mean BAL pepsin values between the two groups. Differences in individual means were calculated by Student's t test for continuous data, and chi squared for categorical data.

	Cases (n=31)	Controls (n=7)	p value
Age (mean, [range])	10.4 [0.4-17.7]	8.3 [3.7-15.1]	0.51
Gender (M/F)	20/11	3/4	0.30
Pepsin values (ng/ml)	24.4 +/- 27.4	4.3 +/- 4	0.03

Table 2: Summary of clinical characteristics, test results and BAL inflammatory indices between the group of CF patients with high pepsin levels and those with low levels. Differences in individual means were calculated by Student's t test or Mann Whitney test for continuous data, and chi-square test for categorical data. PPI: proton pump inhibitor. FEV1: Forced expiratory volume in one second. BMI: Body mass index. NS: Not significant.

	High Pepsin (n=19)	Low Pepsin (n=12)	p value
Age (mean)	10.73	10.07	0.79
Gender (M/F)	13/6	7/5	0.53
Neutrophils ($\times 10^6$ /ml BAL)	5.52	2.61	0.15
BMI z score	-0.94	-0.7	0.66
FEV1 (% predicted)	63	68.4	0.73
BAL NE activity (nM)	56	37	0.29
PPI use	6/19 (32%)	3/12 (25%)	0.69
pH probe performed	5/19 (26%)	1/12 (8%)	0.21
positive pH probe	5/5 (100%)	1/1 (100%)	NS
Chronic <i>P. aeruginosa</i> Colonisation	6/19 (32%)	3/12 (25%)	0.69

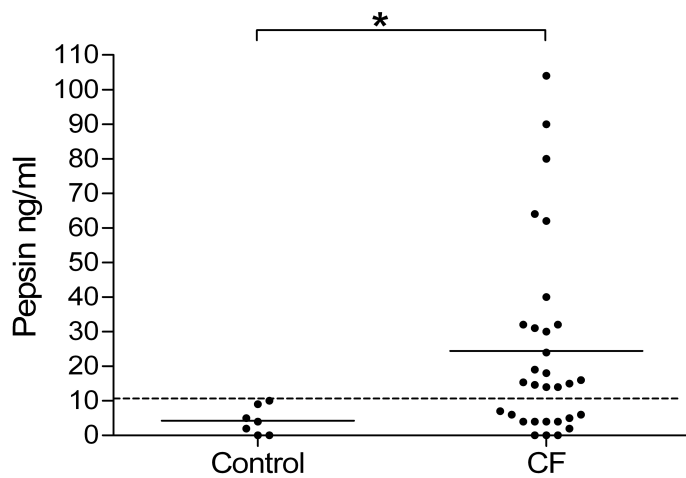


Figure 1: Pepsin levels in BAL from controls and patients with CF. The dotted line (10.4ng/ml) represents the 95th percentile value in the controls. Differences in individual means were assessed by Student's t test.

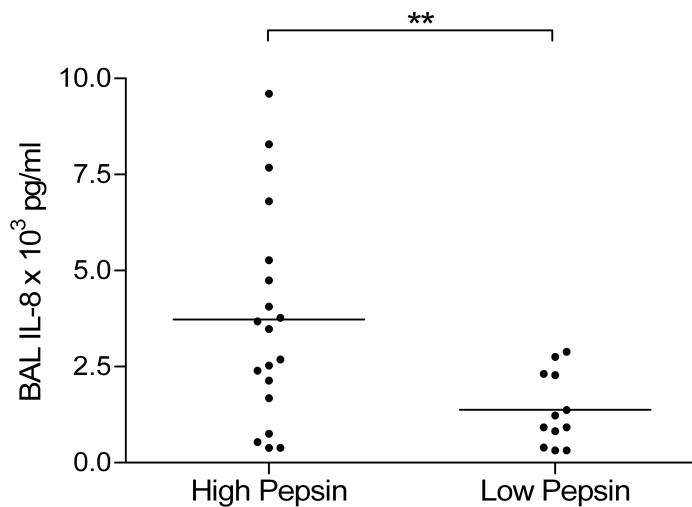


Figure 2: BAL IL-8 levels in CF patients with high and low BAL pepsin values. Differences in means were analysed by Student's t test.

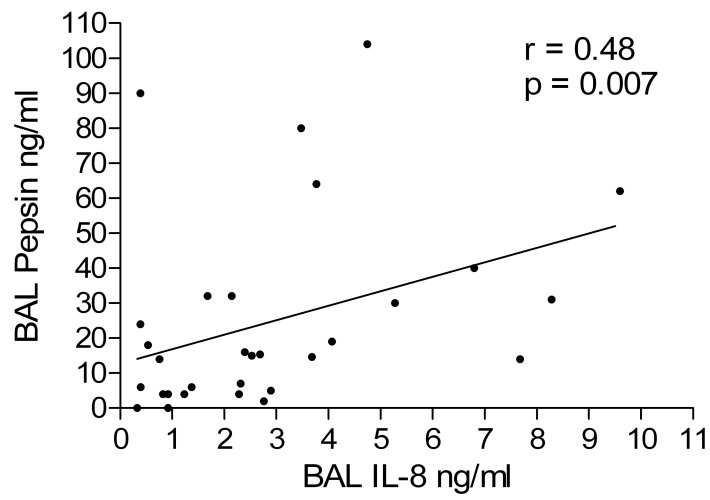


Figure 3: Correlation between BAL pepsin level and BAL IL-8 among patients with CF. Correlation was determined by Pearson's correlation coefficient.