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Evolution of pulmonary inflammation and nutritional status in infants and young children with cystic fibrosis

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ABSTRACT
Introduction Improved nutrition is the major proven benefit of newborn screening programmes for cystic fibrosis (CF) and is associated with better clinical outcomes. It was hypothesised that early pulmonary inflammation and infection in infants with CF is associated with worse nutrition.

Methods Weight, height and pulmonary inflammation and infection in bronchoalveolar lavage (BAL) were assessed shortly after diagnosis in infants with CF and again at 1, 2 and 3 years of age. Body mass index (BMI) was expressed as z-scores. Inflammatory cells and cytokines (interleukin 1β (IL-1β), IL-6, IL-8 and tumour necrosis factor α (TNFα)), free neutrophil elastase activity and myeloperoxidase were measured in BAL. Mixed effects modelling was used to assess longitudinal associations between pulmonary inflammation, pulmonary infection (Staphylococcus aureus and Pseudomonas aeruginosa) and BMI z-score after adjusting for potential confounding factors.

Results Forty-two infants were studied (16 (38%) male; 39 (93%) pancreatic insufficient); 36 were diagnosed by newborn screening (at median age 4 weeks) and six by early clinical diagnosis (meconium ileus). Thirty-one (74%) received antistaphylococcal antibiotics. More than two-thirds were asymptomatic at each assessment. Mean BMI z-scores were −1.5 at diagnosis and 0.5, −0.2 and −0.1 at 1, 2 and 3 years, respectively. Neutrophil elastase and infection with S aureus were associated with lower BMI, whereas age (p=0.01) and antistaphylococcal antibiotics (p=0.013) were associated with increased BMI. On average, each log10 increase in free neutrophil elastase activity was associated with a 0.43 (95% CI 0.06 to 0.79) reduction in BMI z-score.

Discussion Early nutritional status is associated with the underlying pulmonary pathophysiology in CF, and better understanding of these relationships is required. Studies are required to assess whether interventions can decrease pulmonary inflammation and improve nutrition. Early surveillance will enable such targeted interventions with the aim of improving these important clinical outcomes.

INTRODUCTION
Cystic fibrosis (CF) is a common inherited condition that results in a shortened life span. Outcomes for those with CF have consistently improved over the last 40 years such that the median life expectancy is predicted to have reached 50 years for those born after 2000. Much of this improvement is due to aggressive treatment of pulmonary infections and closer attention to maintenance of nutritional status. Many countries have now incorporated newborn screening programmes for CF, and attention is focusing on improving quality and extent of life by earlier intervention in children.

Most of the morbidity and mortality in CF results from progressive suppurative lung disease. Bronchiectasis leads eventually to respiratory failure. Pulmonary inflammation starts early in infancy in those with CF and is exaggerated in response to pulmonary infection. Structural changes also occur much earlier in life than previously thought and, even though those diagnosed by newborn screening appear to have normal lung function during the first 6 months of life, bronchiectasis can be detected shortly after diagnosis. These data are important as they suggest preventing deterioration in lung structure and lung function requires targeted interventions towards improving respiratory health and nutrition as soon as possible after diagnosis, with monitoring of outcomes during the preschool years before pulmonary disease has become extensive.

We have developed a clinically based respiratory early surveillance programme for the detection of lung disease in infants and young children with CF that involves measurement of pulmonary inflammation and infection commencing soon after diagnosis in infants, usually prior to the development of symptoms. As improved nutrition is considered to be associated with better long-term outcomes in CF, and is the major proven benefit arising from newborn screening programmes, we aimed to describe the longitudinal course of pulmonary inflammation from diagnosis following newborn screening over the first 5 years of life and its relationship to the clinically relevant outcome of nutritional status. Specifically, we hypothesised that pulmonary inflammation in infants and young children with CF would be associated with lower body mass index (BMI).

METHODS
Subjects Infants and young children with CF were recruited from the CF clinic of the Princess Margaret Hospital for Children, Perth where an established newborn screening programme has existed since 2000. Briefly, this involves the measurement of
immunoreactive trypsinogen from a heel prick test performed in the first 48–72 h of life. Those with immunoreactive trypsinogen >99th centile undergo cystic fibrosis transmembrane regulator gene mutation analysis. Infants are diagnosed with CF if they are found to be homozygous (or compound heterozygous) for known disease-producing mutations in the CF gene. Infants with one CF-associated gene are referred for sweat testing. Infants were included in the study if they were diagnosed with CF after 2000 by newborn screening, and/or had a positive sweat test. Infants with an early clinical diagnosis (<4 weeks) were also included. Infants were assessed shortly after diagnosis, then around the time of their first birthday and annually thereafter at 2 and 3 years of age. Each visit involved assessment of clinical and nutritional status and bronchoalveolar lavage (BAL). Clinic-recommended policy is for children to use amoxicillin clavulanate as antistaphylococcal prophylaxis during the first 2 years of life.

The surveillance programme was approved by the research ethics committee of The Princess Margaret Hospital for Children, Perth. Written consent was obtained from the parents of all participating subjects for use of clinical data and use of excess BAL and blood samples for research.

**Clinical and nutritional data**

Weight and crown–heel length were measured on the day of testing and expressed as weight, height and BMI z-scores using international growth reference data. A physical examination was performed by a clinician prior to bronchoscopy and BAL. Mode of presentation, genotype, current respiratory symptoms, antibiotic use since the previous assessment and all available microbiology results were reviewed and recorded.

**Bronchoalveolar lavage**

Flexible bronchoscopy was performed under general anaesthesia as previously described with a fibre-optic video-bronchoscope (Olympus XP160F) via an endotracheal tube or laryngeal mask as appropriate. Suction was not performed until the tip of the bronchoscope was past the carina. The BAL involved the instillation of 1 ml/kg of sterile normal saline per aliquot and was retrieved using low pressure suction. Three lavages were performed in the right middle lobe, and one in the lingula or most affected lobe on chest CT. The first sample from each side was sent for microbiological assessment, while the second and third samples from the right side were pooled and analysed for inflammatory markers following centrifugation for 5 min at 1500 rpm. The cell pellet was washed if required, resuspended in 1 ml of phosphate-buffered saline (PBS) and cell counts were conducted. A blinded observer then counted 500 cells and the proportion and number of neutrophils were recorded.

Analysis of inflammatory mediators interleukin 1β (IL-1β), IL-6 and tumour necrosis factor α (TNFα) was conducted using a standard cytokometric bead array human inflammation kit (BD Biosciences, San Diego, California, USA) with a working range between 20 and 5000 pg/ml. Analysis of IL-8 was completed using an ELISA (BD Opt EIA, BD Biosciences, San Diego, California, USA) with a working range between 0.01 and 6.40 ng/ml. Myeloperoxidase (MPO) was measured using an in-house ELISA with a working range between 20 and 5000 pg/ml. Analysis of IL-8 was completed using an ELISA Biosciences, San Diego, California, USA) with a working range between 20 and 5000 pg/ml. Analysis of IL-8 was completed using an ELISA (BD Opt EIA, BD Biosciences, San Diego, California, USA) with a working range between 0.01 and 6.40 ng/ml. Myeloperoxidase (MPO) was measured using an in-house ELISA with a working range between 20 and 5000 pg/ml. Activity was read immediately at 405 nm. The plate was then incubated at 57°C in a CO₂ incubator and read again at 20, 50 and 40 min. The results were calculated using AssayZap and the best time point was taken. The lower limit of detection for this assay was 0.2 μg/ml.

Microbiological samples were cultured on agar plates in the following manner: blood agar, cytosine lactose electrolyte-deficient agar, and blood agar + ticarcillin (for resistant *Pseudomonas aeruginosa* identification) then incubated at 35°C for 48 h in a CO₂ incubator; a PC plate (selective plate for *Burkholderia cepacia* complex) was incubated at 35°C for 48 h in a CO₂ incubator, then at room temperature for a further 24 h; mannitol salt agar for *Staphylococcus aureus* was incubated at 35°C for 48 h in an aerobic atmosphere; Sabarouds agar (for fungi isolation) was incubated at 35°C for 48 h, then at 28°C for a further 14 days; *Fildes* agar (for *Haemophilus* isolation) was incubated at 35°C for 48 h in an anaerobic environment (to prevent overgrowth by *P aeruginosa*). Bacteria were identified by colony morphology, Gram stain and biochemical tests including oxidase and the CS300 screening test specifically for *Pseudomonas*. Microbiological results were reported as positive if at least 10⁶ colony-forming units were identified per ml. Respiratory viruses (respiratory syncitial virus (RSV), parainfluenza 1, 2 and 3, influenza A and B, adenovirus and cytomegalovirus) were detected using direct immunofluorescence and/or rapid viral tissue culture.

**Statistical analysis**

Spearman rank correlation was used to assess bivariate cross-sectional associations between each measure of pulmonary inflammation and BMI z-score. For longitudinal analyses, age (in decimal years) was centred around the mean, and continuous variables were log₁₀ transformed to approximate the normal distribution. Due to the small number of samples and incomplete follow-up data, linear mixed effects analysis, with random intercept for subjects, was applied to assess longitudinal associations between BMI z-score and measures of pulmonary inflammation. Models were also adjusted for age, gender, anti-staphylococcal antibiotics and presence of symptoms. Linear mixed effects modelling was done in Stata version 10.0, and all other analyses were done through SPSS 15. For the analyses, values for 50% of the lowest limits of detection were substituted where pulmonary inflammation was undetected in BAL.

**RESULTS**

Forty-two infants (16 (38.1%) male) were diagnosed with CF between the inception of newborn screening and February 2006. This period was chosen to allow for longitudinal data collected up to the age of ~3 years to be included. Thirty-six of these were diagnosed by newborn screening (at median age 4 weeks) and six by early clinical diagnosis (all with meconium ileus diagnosed <4 weeks). Twenty-four (57%) were homozygous and 16 (38%) heterozygous for the Phe508del mutation, respectively. Thirty-nine (92.9%) infants were pancreatic insufficient. Parent-reported respiratory symptoms (cough, wheeze or any breathing difficulty) were present at the time of the assessments in 38, 36, 31 and 36%, respectively, of the 36 infants diagnosed by newborn screening. A total of 40, 77, 63 and 57% of infants, respectively, were receiving prophylactic antistaphylococcal antibiotics at assessment.

**Nutritional indices**

Height was well preserved during the course of the study. Median weight z-scores were reduced at diagnosis but increased and normalised during subsequent evaluations. BMI
z-scores followed a similar pattern to weight z-scores (figure 1 and table 1).

**Association between pulmonary inflammation and BMI z-score**

Pulmonary inflammation identified at each assessment is shown in table 1. Neutrophils were detected in all BAL samples. There were no obvious trends over time in the proportion of neutrophils in the BAL total cell population or in the number of neutrophils in the BAL. IL-1β, IL-6 and MPO were detected in most BAL samples and no obvious trend could be identified at the different assessments. NE was detected in approximately a third of BAL and less frequently than IL-8 which was detected in ~80% of BAL (table 1). When detected, levels of NE increased over the first 2 years but then fell slightly at the final BAL, whereas IL-8 increased throughout the study (table 1 and figure 2).

Significant correlations were seen between the different indices of neutrophilic inflammation: neutrophil number (and proportion), IL-8, NE and MPO. Thus, the effects of inflammation variables on BMI were examined individually. There was no obvious correlation between pulmonary inflammation and BMI z-score at any of the individual assessments (correlations shown for NE and IL-8 in table E1 of the online supplement). In longitudinal analyses (table 2), log_{10} neutrophil number, log_{10} IL-6, log_{10} IL-8, log_{10} MPO, log_{10} IL-1β and log_{10} TNFα were not significantly associated with reduced BMI z-score, whereas log_{10} free NE was associated with a lower BMI z-score (p=0.022). On average, each log_{10} increase in NE was associated with a 0.45 (95% CI 0.06 to 0.79) reduction in BMI z-score, as shown in table 2. Details of the full adjusted mixed effects model for the association between NE and BMI z-score is shown in table E2 of the online supplement.

Age (p=0.01) and treatment with antistaphylococcal antibiotic prophylaxis (p=0.013) had a significant positive association with BMI z-score, whereas gender and presence of symptoms were not associated with BMI z-score.

**Association between pulmonary infection and BMI z-score**

Apart from one infant in whom RSV was isolated, viruses were not detected in BAL. The known CF airway pathogens, *S aureus* and *P aeruginosa*, were the most commonly detected bacteria in BAL (table 1). Other organisms detected in the respective BAL were in the first BAL: *Escherichia coli* (n=1), *Aspergillus fumigatus* (n=1), *Haemophilus influenzae* (n=1), *Enterobacter cloacae* (n=4), *Klebsiella* spp. (n=1); in the second BAL: *A fumigatus* (n=1), *H influenzae* (n=1), *E cloacae* (n=1); in the third BAL: *A fumigatus* (n=1), *Mucorrella catarrhalis* (n=1), *Stenotrophomonas maltophilia* (n=1); in the fourth BAL: *E coli* (n=1), *A fumigata* (n=1), *H influenzae* (n=3), *Klebsiella* spp. (n=1).

A mixed effects analysis was used to evaluate the influence of pulmonary infection (*S aureus* and *P aeruginosa*) on BMI z-score after adjusting for potential confounders (table 3). Infection with *S aureus* was associated with a mean reduction of 1.04 z-score for BMI. No association was observed for infection with *P aeruginosa* (p=0.918). Thus, respiratory tract infection with *S aureus* identified in BAL was associated with lower BMI z-scores after accounting for age, gender and antistaphylococcal antibiotic prophylaxis.

Infection with either *S aureus* (mean 0.59; 95% CI 0.12 to 0.66; p=0.004) or *P aeruginosa* (mean 0.78; 95% CI 0.46 to 1.09; p<0.001) was significantly associated with increased log_{10} free NE, but treatment with antistaphylococcal antibiotic prophylaxis was not (−0.021; 95% CI −0.226 to 0.185; p=0.842).

**DISCUSSION**

Many countries in Europe and the majority of the USA have now adopted newborn screening programmes for CF. Improved nutritional status is an important clinical outcome in CF and is the most obvious benefit afforded by an early diagnosis from newborn screening. In the present single-centre study in infants and young children with CF diagnosed mainly by newborn screening we have shown that early pulmonary inflammation characterised by free NE and infection with *S aureus* are associated with worse nutritional status in the first few years of life, in the absence of clinically apparent respiratory disease in most children. This association was evident despite modern CF care that included an aggressive clinical approach to improving nutrition, apparent normalisation of nutritional status in the vast majority of subjects and treatment of any pulmonary exacerbations. The results of our study are crucial in informing future clinical practice if further benefits from newborn screening are to be realised.

Several studies have demonstrated that pulmonary inflammation is evident soon after diagnosis in CF, but the longitudinal nature of our study allowed us to investigate its relationship with BMI over the first 3 years of life. This is important as any relationship between pulmonary inflammation and BMI z-score is likely to be temporally complex. Assessment of inflammation in the BAL provides a single-time ‘snap-shot’ of the inflammatory processes occurring in the lungs. However, BMI provides a cumulative assessment of nutrition. Thus the lack of correlation between these at single time points is not surprising. BMI

![Figure 1](image-url)  
**Figure 1** (A) Height, (B) weight and (C) body mass index (BMI) z-scores measured soon after diagnosis and at 1, 2 and 3 years of age. The box and whiskers represent the median, IQR and range respectively.
In the post-screening era, where infants generally had fewer cough symptoms, better lung function and better nutritional status such as measurement of body composition during the first few years of life could have further significant positive health benefits in later life for those with CF. Although treating fat malabsorption due to pancreatic insufficiency results in apparent normalisation of nutritional status in the majority, our study highlights the need to investigate the association between pulmonary inflammation and nutritional status further in order to find new strategies for optimising clinical outcomes.

We measured cytokines, cells and inflammatory products known to be associated with pulmonary disease in CF. We found that NE was significantly associated with a reduction in BMI.

z-scores or percentiles have been shown to be a valid measure of nutritional status in CF and predict nutritional failure more sensitively and accurately than conventional anthropometric measures. In the post-screening era, where infants generally maintain normal height in contrast to children diagnosed following clinical presentation, we believe that BMI z-score can be used instead of other measures of nutritional status such as weight-for-height. However, more comprehensive measures of nutritional status such as measurement of body composition may provide a better insight into early nutritional status and should be considered for future studies. In older subjects, improved nutritional status has been shown to be associated with improved survival in those with CF. In childhood, increased BMI is associated with better lung function. Recent data from the Wisconsin group identified that patients with CF who achieved early growth recovery within 2 years of diagnosis had fewer cough symptoms, better lung function and better chest radiograph scores at 6 years of age, whereas growth patterns between the ages of 2 and 6 years were not associated with pulmonary measures at age 6. Their finding that benefits of newborn screening on pulmonary status at 6 years of age depend on early nutritional outcomes emphasises the need for comprehensive and aggressive treatment implemented as soon as possible after diagnosis. Thus, it is likely that improving BMI during the first few years of life could have further significant positive health benefits in later life for those with CF.

We measured cytokines, cells and inflammatory products known to be associated with pulmonary disease in CF. We found that NE was significantly associated with a reduction in BMI.
Table 2  Adjusted associations between measures of pulmonary inflammation and BMI z-score

<table>
<thead>
<tr>
<th>Log10 of variables</th>
<th>Change in BMI z-score</th>
<th>SE</th>
<th>p Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free NE</td>
<td>−0.43</td>
<td>0.19</td>
<td>0.022</td>
<td>−0.79 to −0.06</td>
</tr>
<tr>
<td>Neutrophil number</td>
<td>−0.23</td>
<td>0.19</td>
<td>0.224</td>
<td>−0.60 to 0.14</td>
</tr>
<tr>
<td>IL-6</td>
<td>−0.22</td>
<td>0.27</td>
<td>0.41</td>
<td>−0.74 to 0.30</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.46</td>
<td>1.28</td>
<td>0.721</td>
<td>−2.05 to 2.96</td>
</tr>
<tr>
<td>MPD</td>
<td>0.20</td>
<td>0.22</td>
<td>0.356</td>
<td>−0.63 to 0.23</td>
</tr>
<tr>
<td>IL-1β</td>
<td>−0.25</td>
<td>0.24</td>
<td>0.293</td>
<td>−0.72 to 0.22</td>
</tr>
<tr>
<td>IL-8</td>
<td>−0.18</td>
<td>0.16</td>
<td>0.271</td>
<td>−0.50 to 0.14</td>
</tr>
</tbody>
</table>

Data shown represent independent associations for each measure of inflammation with BMI. Models were adjusted for gender, antistaphylococcal antibiotic prophylaxis, presence of respiratory symptoms and centred-age squared.

BMI, body mass index; IL, interleukin; MPD, myeloperoxidase; NE, neutrophil elastase; TNF, tumour necrosis factor.

Table 3  Associations between pulmonary inflammation and body mass index (BMI) z-score

<table>
<thead>
<tr>
<th>Organism</th>
<th>Change in BMI z-score</th>
<th>SE</th>
<th>p Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>S aureus</td>
<td>−1.04</td>
<td>0.31</td>
<td>0.001</td>
<td>−1.64 to −0.43</td>
</tr>
<tr>
<td>P aeruginosa</td>
<td>0.04</td>
<td>0.36</td>
<td>0.918</td>
<td>−0.66 to 0.74</td>
</tr>
</tbody>
</table>

The model was adjusted for gender, antistaphylococcal antibiotic prophylaxis, and centred-age squared.

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Competing interests  None.

Contributors  Conceived and designed the study: SCR, SMS, PDS. Laboratory analysis: SB. Database development, data entry and handling: CG. Data analysis: SCR.
Genetic variants and risk of lung cancer in never smokers

This four-stage study systematically investigated common genetic variations associated with the risk of developing lung cancer in never smokers. Stage 1 involved a genome-wide association study at the Mayo Clinic. A total of 331,918 single nucleotide polymorphisms (SNPs) in 377 case-control matched pairs were analysed. The 44 most significant SNPs that might alter the risk of lung cancer in never smokers were selected. The MD Anderson Cancer Center and Harvard School of Public Health (n=735 and 335, respectively) tested these 44 most significant SNPs. The SNP rs2352028 (at 13q31.3) was the one most associated with lung cancer in never smokers. This finding was repeated in a replication cohort.

For all four studies the combined p value for the association between rs2352028 and lung cancer in never smokers was \(5.94 \times 10^{-6}\) (OR 1.46, 95% CI 1.26 to 1.70). 10% of lung cancer cases in never smokers could be attributed to genetic variation of SNP rs2552028. A gene expression profile analysis confirmed that the expression of the gene GPC5 was associated with genotypes of SNPs, with lower expression in high-risk alleles.

This study suggests that genetic variants affecting the expression of the GPC5 gene are responsible for increasing the risk of lung cancer in never smokers. However, it was concluded that the sample sizes used did not allow for enough power, and there was inconsistent adjustment for confounders such as second-hand smoke exposure across the different centres. GPC5 gene alterations have previously been shown to influence lung cancer development and merit further study.


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