Upper and lower airway nitric oxide levels in primary ciliary dyskinesia, cystic fibrosis and asthma

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Summary
Background: Patients with primary ciliary dyskinesia (PCD) have abnormal ciliary function and low nitric oxide levels. Nitric oxide (NO) biosynthesis is dependent on nitric oxide synthases (NOS). Cilia line the bronchial but not the alveolar epithelium. It has been hypothesised that NOS function relies on normal ciliary function and that in PCD bronchial but not alveolar NO might therefore be reduced. The aim of this study was to assess bronchial and alveolar NO levels primarily comparing healthy children to PCD and secondarily to cystic fibrosis (CF) and asthmatic children.

Methods: Multiple flow-rate fractional exhaled and nasal NO measurements were performed using a NIOX® Flex NO analyser (Aerocrine, Sweden) in children with PCD (n = 14), asthma (n = 18), CF (n = 12) and healthy controls (n = 18). Alveolar and bronchial NO levels were derived using a model of pulmonary NO exchange-dynamics.

Results: Both the bronchial and alveolar NO were significantly lower in PCD than healthy controls (mean (SD) 264 (209) picolitres/second (pl/s) vs. 720 (514) pl/s, p = 0.024 and 1.7 (0.8) parts per billion (ppb) vs. 3.5 (1.3) ppb, p = 0.001 respectively.) In asthmatics bronchial NO was found to be significantly higher than in healthy controls and in children with CF alveolar NO was significantly lower (2100 (1935) pl/s, p = 0.045 and 2.5 (1.2) ppb, p = 0.034 respectively.)

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Introduction

Nitric oxide is a key cellular signalling molecule, involved in diverse physiological and pathophysiological processes such as vascular homeostasis, immune cell activity and bronchomotor tone.\(^1\) It is synthesized in the respiratory epithelium from L-arginine by three NO synthase isoforms (NOS).\(^2\) Infection and inflammation lead to inducible NOS stimulation and hence NO biosynthesis.\(^2\) Airway NO, can be measured using simple non-invasive techniques via the reaction of NO with ozone and subsequent detection by chemiluminescence through commercially available equipment.\(^3\) This allows measurement of nasal NO (nNO) and fractional exhaled NO, typically measured at a flow rate of 50 ml/s (FeNO50).

Primary ciliary dyskinesia (PCD) is an autosomal recessive condition in which abnormal ciliary structure and/or function lead to impaired mucociliary clearance, recurrent sino-pulmonary infection and, ultimately, bronchiectasis.\(^4\) Nasal NO and, to a lesser extent, FeNO are characteristically low in PCD, so much so that nNO is used as a screening test for PCD in many specialist centres.\(^5\) Abnormal NO concentrations have been identified in a number of other respiratory diseases including cystic fibrosis (CF), where NO levels are reduced compared to healthy controls but not to the degree seen in PCD and asthma where airway levels are elevated.\(^6,7\)

Localising the relative contributions of different areas of the lung to total FeNO levels in these conditions would increase our understanding of the underlying pathophysiology, potentially providing a useful non-invasive marker for disease progression, and possibly help to tailor therapies. A two-compartment model of pulmonary NO exchange dynamics, using FeNO measurements at several different flow-rates, allows estimates of the relative contribution of the bronchial (J′awNO) and alveolar (CalvNO) areas of the lung to total FeNO.\(^8,9\) Bronchial NO flux is calculated as the product of the bronchial wall NO concentration and the NO diffusing capacity of the airway.\(^7\) The alveolar NO concentration is calculated as a steady NO source, a balance between locally biosynthesised and inhaled NO vs NO diffusion away.\(^8,9\)

The raised NO levels seen in asthma are attributed to eosinophilic inflammation however, it is unclear why NO is low in PCD and CF despite persistent airway infection and inflammation.\(^10\) A number of potential mechanisms for the extremely low NO in PCD have been proposed (reviewed in Ref.\(^11\)) however, to date, the actual mechanism(s) remains elusive. Several papers have hypothesised that it may result from a reliance of normal NOS activity, and hence NO biosynthesis, on normal ciliary ultrastructure and/or function due to a mechano-chemical coupling to dynein ATPases in the ciliary axoneme.\(^12,13\) If this hypothesis were correct one would expect bronchial NO to be reduced in PCD, as cilia are present in the bronchial compartment and ciliary function is impaired in PCD. However cilia are not present in the alveolar compartment, so alveolar NO should not be affected by impaired ciliary function and might be comparable with healthy controls.

Conclusion: Our findings do not support the hypothesis that NOS and ciliary function are coupled instead suggesting a more generalised mechanism for the low levels of NO seen in PCD. Our findings in CF and asthma corroborate evidence that these are diseases of the lung peripheries and bronchi respectively.

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Methods

This study was approved by Southampton and South West Hampshire Research Ethics Committee (A). REC numbers: 06/Q1702/109 and 08/H0502/126. All subjects gave written informed consent.

Participants

Children with PCD (n = 14), asthma (n = 18) and CF (n = 12) patients were recruited from specialist PCD and paediatric respiratory clinics. Healthy children (n = 18) recruited from non-respiratory clinics completed a short questionnaire to exclude disease that might affect nitric oxide levels. PCD was diagnosed by assessing the ciliary beat frequency and pattern of airway epithelial cells by high-speed video microscopy in patients with a suggestive history. Diagnosis was further supported by assessment of ciliary ultrastructure by transmission electron microscopy and, in some cases, analysis of re-differentiated cilia following culture of the airway epithelial cells at an air liquid interface.\(^4\) CF diagnosis was based on compatible history, an abnormal sweat test and/or CF genotyping. Asthma diagnosis was based on clinical history and characteristic spirometry with reversibility.\(^16\) Children with asthma were on different British Thoracic Society asthma management steps: step 1 (n = 1), step 2 (n = 2), step 3 (n = 7), step 4 (n = 5), step 5 (n = 3).\(^16\) CF, asthma and healthy children were age and sex matched to the participants with PCD.

For inclusion the children had to be over 6 years of age and be well on the day of testing with no evidence of respiratory tract infection in the previous four weeks. They were excluded if they had multiple respiratory diagnoses, had smoked in the last year or had undertaken spirometry in the previous hour. We also assessed 14 healthy adults in order to optimise the methods (data shown in Supplementary Table 1).

Measurement of airway nitric oxide

Measurement of nasal and lower airway nitric oxide levels using a chemiluminescent NO analyser, NIOX® Flex (Aerocline, Sweden) followed American Thoracic Society/European Respiratory Society (ATS/ERS) recommendations.\(^17\)
Nasal NO was measured during a breath holding manoeuvre to close the velum whilst a nasal probe sampled gas aspirated from the nostril at a rate of 5 ml/s. Patients were encouraged to hold each breath for approximately 20 s until the analyser recorded a plateau in nitric oxide concentrated from the aspirated gas. Three measurements were obtained from each child using the same nostril and the mean nNO reading was recorded.

FeNO was measured at multiple flow-rates (50, 100, 200 and 250 ml/s) whilst maintaining a constant exhalation pressure &gt;5 cm H2O through visual feedback. Two consistent readings (within 10%) were obtained at each flow-rates. Calculation of CalvNO (ppb) and J'awNO (nl/min) were based on the mathematical model of pulmonary NO exchange dynamics proposed by Tsoukias & George9:

\[ V_{NO} = V_E \times CalvNO + J'awNO \]

Where NO elimination (\( V'_{NO} \)) (nl/s) is the exhaled NO concentration (ppb) \&times flow rate (\( V'_E \)) (ml/s), J'awNO (pl/s) is bronchial NO flux, and CalvNO (ppb) is the steady-state NO concentration in alveolar air. Therefore the gradient and intercept of a regression line on a graph of NO elimination (\( V'_{NO} \)) against flow-rate (\( V'_E \)) represent CalvNO and J'awNO respectively (Fig. 1).8,9

Spirometry was measured, following NO measurement, in respiratory participants using a Master Screen™ Body (Jaeger) in accordance with the ATS guidelines.18

Statistical analyses

The natural log of J'awNO, CalvNO and FeNOSO were found to be normally distributed hence were used for statistical analysis. Non-parametric analysis was used for nNO. Least squares regression models were used to evaluate univariate relationships between the NO parameters and independent variables: age, sex, height, weight, FEV1, ambient NO, use of inhaled corticosteroids and antibiotics. If log transformation was necessary to achieve normal distribution, log transformed data were used in the model. Linear regression analysis and a standard backwards model selection process were used in order to assess relationships between these variables and NO parameters and to identify confounding variables. Analysis of Variance (ANOVA) was used to compare the geometric mean of J'awNO, CalvNO and FeNOSO between respiratory disease groups. FeNOSO and subject demographics were also compared between groups using the ANOVA. Nasal NO was compared between groups using the Kruskal–Wallis test. Confounding variables were adjusted for during the analyses. A statistical significance level of 0.05 was used throughout. The data were evaluated using statistical analysis software SPSS version 19.0.0 (IBM, USA).

Results

There were no differences seen in the demographics of children in the different respiratory groups apart from lower weight in those with CF compared to healthy controls (Table 1).

All NO measurements were completed by the participants apart from one child with PCD who was unable to perform the multiple-flow FeNO protocol and three with asthma who were unable to perform nNO measurements due to nasal obstruction.

Primary ciliary dyskinesia

As expected nNO was significantly lower in children with PCD compared to healthy control children (median (inter-quartile range) 27 (16–76) ppb vs. 772 (690–886) ppb, \( p &lt; 0.001 \)) (Table 2 & Fig. 2a). Both bronchial and alveolar NO were significantly lower in children with PCD compared to healthy controls (mean (SD) 264 (209) pl/s vs. 720 (514) pl/s, \( p = 0.024 \), and 1.7 (0.8) ppb vs. 3.5 (1.3) ppb, \( p = 0.001 \), respectively) (Table 2 & Fig. 2b). FeNOSO was also lower in children with PCD, but not with significance (mean (SD) 8.8 (7.3) ppb vs. 16.7 (10.8) ppb, \( p = 0.062 \)) (Table 2 & Fig. 2b).

Cystic fibrosis

There was no significant difference in nNO, FeNOSO or bronchial NO between children with CF and healthy controls (Table 2, Fig. 2a–c). However, alveolar NO was significantly lower in children with CF (2.5 (1.2) ppb vs. 3.5 (1.3) ppb, \( p = 0.001 \)) (Table 2 & Fig. 2d).

Asthma

There was no significant difference in nNO, FeNOSO or alveolar NO between asthma and healthy controls (Table 2, Fig. 2a, b and d). However, bronchial NO was significantly higher in asthmatics (2100 (1935) pl/s vs. 720 (514) pl/s, \( p = 0.045 \)) (Table 2 & Fig. 2c).

Relationships between NO parameters and independent variables

There was no significant relationship between parameters NO and FEV1, FVC or FEF25–75. Ambient NO was 6.0 (7.9) ppb (mean (SD)) and no relationship was seen between this and any other NO parameters.
Discussion

The two-compartment model has been validated in an over 20 studies across different respiratory disease groups and carries the advantage over standard FeNO measurements in allowing assessment of NO biosynthesis specifically from the bronchial and alveolar compartments of the lung.8,14,15,19 To our knowledge, this is the first study directly comparing these parameters in PCD, CF, asthma and healthy subjects within the same study thereby negating issues of methodological variation between studies when comparing these different respiratory disease groups.

In our population of children we have demonstrated that both alveolar and bronchial NO levels are reduced in PCD while only alveolar levels are reduced in CF and in asthma bronchial NO levels are raised.

Upper airway nitric oxide levels

As previously reported, we found nasal NO levels to be extremely low in PCD and reduced in CF compared to healthy and asthmatic children.11,23

Lower airway nitric oxide levels in primary ciliary dyskinesia

Our data do not support the study hypothesis that low levels of airway NO in PCD are due to NOS activity requiring normal ciliary function.12,13 Also opposing the hypothesis, different PCD phenotypes including static and hyper-frequent, had similarly low levels of NO. Instead our findings point to a more generalized mechanism that is not localized to a specific lung compartment. There are a number of proposed mechanisms for this including: 1) reduced biosynthesis of NO due to reduced NOS activity11; or 2) increased NO breakdown to its metabolites.11 Three studies have utilized this two-compartment model to assess bronchial and alveolar NO concentrations in PCD.13–15 Consistent with our findings, all three studies demonstrated low bronchial NO in PCD patients compared to controls however only Mahut et al. also found low alveolar NO.13–15 Of the other two studies that found equivalent alveolar NO levels between PCD and controls, the work by Shoemark et al. was undertaken in adults.13 Interestingly when we compared our adult healthy control group (assessed to optimize the methods) to our PCD group

Table 1 Demographics in subjects with primary ciliary dyskinesia, cystic fibrosis and asthma compared with healthy controls (* indicates p < 0.05) (data presented as mean (SD) unless stated).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group</th>
<th>Healthy controls</th>
<th>PCD</th>
<th>CF</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants</td>
<td>18</td>
<td>14</td>
<td>12</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Males (n) (%)</td>
<td>10 (56)</td>
<td>6 (46)</td>
<td>6 (50)</td>
<td>6 (33)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>14.1 (2.3)</td>
<td>12.8 (3.9)</td>
<td>11.7 (3.1)</td>
<td>13.5 (3.5)</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161 (14)</td>
<td>155 (23)</td>
<td>145 (17)</td>
<td>154 (17)</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>63 (24)</td>
<td>48 (21)</td>
<td>39 (12)*</td>
<td>52 (19)</td>
<td></td>
</tr>
<tr>
<td>FEV1 (z-score)</td>
<td>–</td>
<td>–0.89 (1.00)</td>
<td>–1.15 (1.2)</td>
<td>0.35 (1.39)</td>
<td></td>
</tr>
<tr>
<td>FVC (z-score)</td>
<td>–</td>
<td>–0.76 (1.64)</td>
<td>–0.60 (0.94)</td>
<td>0.28 (1.10)</td>
<td></td>
</tr>
<tr>
<td>FEF25–75 (z-score)</td>
<td>–</td>
<td>–1.39 (1.13)</td>
<td>–1.98 (1.05)</td>
<td>–1.39 (1.13)</td>
<td></td>
</tr>
<tr>
<td>Inhaled corticosteroids (n) (%)</td>
<td>0</td>
<td>3 (21)</td>
<td>4 (33)</td>
<td>17 (94)</td>
<td></td>
</tr>
<tr>
<td>Antibiotics (n) (%)</td>
<td>0</td>
<td>9 (69)</td>
<td>11 (92)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa (n) (%)</td>
<td>–</td>
<td>0 (0)</td>
<td>1 (8)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Other microorganisms (n) (%)</td>
<td>–</td>
<td>5 (38)</td>
<td>3 (25)</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

Data unavailable.

Table 2 Nitric oxide parameters in subjects with primary ciliary dyskinesia, cystic fibrosis and asthma compared with healthy controls (* & ** indicate p < 0.05 & p < 0.001 respectively) (data presented as mean (SD) unless stated).

<table>
<thead>
<tr>
<th>Nitric oxide parameter</th>
<th>Group</th>
<th>Healthy controls</th>
<th>PCD</th>
<th>Cystic fibrosis</th>
<th>Asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNO (ppb)*</td>
<td>772 (690–886)</td>
<td>27 (16–76)**</td>
<td>501 (450–608)</td>
<td>769 (560–1124)c</td>
<td></td>
</tr>
<tr>
<td>FeNO3 (ppb)</td>
<td>16.7 (10.8)</td>
<td>8.8 (7.3)</td>
<td>14.4 (11.2)</td>
<td>43.4 (41.2)b</td>
<td></td>
</tr>
<tr>
<td>J'awNO (pl/s)</td>
<td>720 (514)</td>
<td>264 (209)</td>
<td>519(495)</td>
<td>2100 (1935)b</td>
<td></td>
</tr>
<tr>
<td>CalvNO (ppb)</td>
<td>3.5 (1.3)</td>
<td>1.7 (0.8)**</td>
<td>2.5 (1.2)*</td>
<td>5.4 (3.5)</td>
<td></td>
</tr>
</tbody>
</table>

* Median (IQR).
| Adjusted for weight.
| n = 15 as 3 patients unable to perform nNO test.
| n = 13 as 1 patient unable to perform FeNO tests.
Supplementary Table 1) we similarly found comparable alveolar NO levels. Paraskakis et al. also found comparable levels of alveolar NO between PCD and controls. However 63% of their PCD group were taking inhaled corticosteroids (ICS) as compared to only 23% of our PCD population and we speculate that a higher rate of asthma co-morbidity might account for the differences in alveolar NO between studies. There may be other differences between the study populations such as concurrent respiratory infection.

Lower airway nitric oxide levels in cystic fibrosis

Consistent with two previous studies we found that bronchial NO in CF was similar to healthy children. We found that alveolar NO was significantly lower in our CF group, consistent with the original work in the area by Shin et al. however contrary to data from Suri et al. who found it to be elevated. We note that there were a number of differences in the clinical characteristics of their CF group to ours, with 50% taking ICS and 23% growing Pseudomonas aeruginosa (33% and 8% respectively in our CF group). Nevertheless, as with the PCD group, the differences between studies may reflect other more complex mechanisms not captured in the available clinical data.

Our findings in the CF group, in contrast to the asthma group and consistent with general opinion, suggest that CF is mainly a disease of the peripheral airway. Given the antimicrobial properties of NO, the low alveolar NO levels might contribute to the chronic recurrent bacterial infections seen in CF. However, alternatively, chronic peripheral lung infection with denitrifying bacteria might lead to increase NO breakdown and hence the low alveolar NO levels and further work in this area is required.

Despite its advantages, the two-compartment model is an idealised representation of pulmonary anatomy and

Figure 2 (a-d) - Box and whisker plot comparisons of (a) nasal nitric oxide concentration (nNO); (b) fractional exhaled nitric oxide concentration at 50 ml/s (FeNO<sub>50</sub>); (c) bronchial nitric oxide flux (J'awNO) and (d) alveolar nitric oxide concentration (CalvNO) in 18 healthy controls, 14 primary ciliary dyskinesia (PCD), 12 cystic fibrosis (CF) and 15 asthma patients. The box represents the interquartile range intersected by the median. The whiskers represent the range of data. Nasal NO is significantly lower in PCD compared to healthy subjects. J'awNO is significantly lower in PCD patients and higher in asthma patients compared to healthy controls. CalvNO is significantly lower in PCD and CF patients compared to healthy controls. (ppb, parts per billion; pl/s, picolitres per second).

Lower airway nitric oxide levels in asthma

There is a large literature on the potential role of FeNO in the pathophysiology and management of asthma. It has generally been found that high FeNO levels are seen in asthma, particularly in untreated asthmatics, and that this is reduced with ICS. This has been demonstrated to originate from the bronchial region whilst alveolar NO remains relatively normal and this is consistent with our data. A recent Cochrane review concluded that FeNO-guided steroid therapy in asthma should not be recommended. However it has been suggested that bronchial NO might be more sensitive than FeNO to monitor response to ICS therapy and that these parameters may also be of benefit for disease phenotyping.
function and has intrinsic limitations. Notably, it fails to account for axial molecular diffusion of bronchial NO back into the alveolar region, which may lead to a spurious rise in alveolar NO and decreased bronchial NO estimates.\textsuperscript{31–33} Nevertheless, our understanding of pulmonary NO exchange is in evolution and the importance of heterogeneous ventilation and inflammation have yet to be confirmed.\textsuperscript{34,35} Larger numbers of subjects would have been of benefit but due to the rarity of PCD, full validation will require multi-centre collaboration. The numbers we have investigated are in keeping with previously reported studies and we included all eligible children with PCD in our clinic.

$FeNO$ was measured according to ATS/ERS guidelines to ensure accuracy and repeatability of readings.\textsuperscript{17} $FeNO$ is non-invasive, acceptable to patients and highly reproducible.\textsuperscript{17} Contamination of $FeNO$ with $nNO$ was avoided by exhalation against a minimum mouthpiece pressure of 5 cm H\textsubscript{2}O.\textsuperscript{17,36} This process causes velum closure has been included in the ERS guidelines.\textsuperscript{38} Similarly we found no relationship taken to control for non-disease-related factors that may have yet to be confirmed.\textsuperscript{34,35} Larger numbers of subjects would have been of benefit but due to the rarity of PCD, full validation will require multi-centre collaboration. The numbers we have investigated are in keeping with previously reported studies and we included all eligible children with PCD in our clinic.

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Due to the significant variance of bronchial and alveolar NO levels within disease groups these parameters will have a limited role in aiding the diagnosis of these respiratory conditions. To date there is little published data on their use in monitoring disease progression and the benefit of therapeutic interventions, however the limited work available in asthma has promise.\textsuperscript{19,24} In CF the standard practice of monitoring spirometry in what is a disease of the lung peripheries and bronchi respectively. While the use of these NO parameters are matched.\textsuperscript{17}

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The potential benefit of using alveolar NO, a non-invasive risk-free investigation, in this role might warrant further investigation.

**Conclusion**

Our findings do not support the hypothesis that NOS and ciliary function are coupled, instead suggesting a more generalised mechanism for the low levels of NO seen in PCD. In our population alveolar NO levels are abnormal in CF and bronchial NO levels in asthma corroborating evidence that these are diseases of the lung peripheries and bronchi respectively. While the use of these NO parameters are unlikely to aid the diagnosis of respiratory disease longitudinal studies may find benefit in their use in monitoring therapeutic interventions and disease progression.

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**Conflict of interest**

The authors declare no conflict of interest.

**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.rmed.2012.11.021.

**References**


