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

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Received 14 August 2012; accepted 28 November 2012

KEYWORDS

Nitric oxide;
Primary ciliary
dyskinesia;
Cystic fibrosis;
Asthma;
Bronchial nitric
oxide;
Alveolar nitric oxide

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.)

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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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 (Fe_{NO50}).

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, Fe_{NO} 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 Fe_{NO} 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 Fe_{NO} measurements at several different flow-rates, allows estimates of the relative contribution of the bronchial (J'_{awNO}) and alveolar ($Calv_{NO}$) areas of the lung to total Fe_{NO} .^{8,9} Bronchial NO flux is calculated as the product of the bronchial wall NO concentration and the NO diffusing capacity of the airway.⁹ 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.

The three studies that have used this two-compartment model in PCD patients to date have conflicting results, but varied in age and demographics of participants and NO analysers used.^{13–15} We are the first single study to assess

the relative contributions of the bronchial and alveolar compartments of the lung to total Fe_{NO} , comparing healthy children to those with PCD and secondarily to children with CF and asthma.

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 (Aerocrine, 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.

Fe_{NO} was measured at multiple flow-rates (50, 100, 200 and 250 ml/s) whilst maintaining a constant exhalation pressure >5 cm H₂O through visual feedback. Two

consistent readings (within 10%) were obtained at each flow-rates. Calculation of Calv_{NO} (ppb) and J'aw_{NO} (nl/min) were based on the mathematical model of pulmonary NO exchange dynamics proposed by Tsoukias & George⁹:

$$V'_{\text{NO}} = V_E \times \text{Calv}_{\text{NO}} + \text{J'aw}_{\text{NO}}$$

Where NO elimination (V'_{NO}) (nl/s) is the exhaled NO concentration (ppb) $\times$ flow rate (V'_E) (ml/s), J'aw_{NO} (pl/s) is bronchial NO flux, and Calv_{NO} (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 Calv_{NO} and J'aw_{NO} 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'aw_{NO} , Calv_{NO} and $\text{Fe}_{\text{NO}50}$ 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, FEV_1 , 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'aw_{NO} , Calv_{NO} and $\text{Fe}_{\text{NO}50}$ between respiratory disease groups. $\text{Fe}_{\text{NO}50}$ 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).

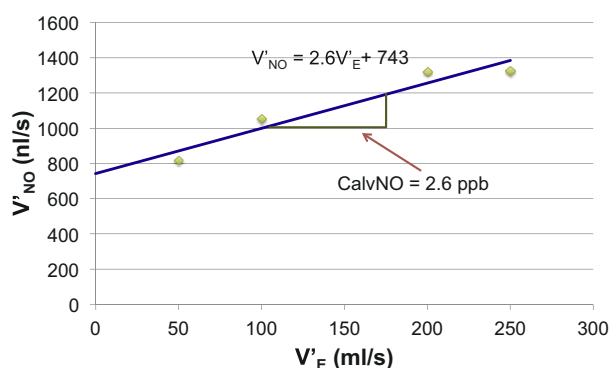


Figure 1 Example of the two-compartment mathematical technique used to estimate bronchial and alveolar contributions to Fe_{NO} . Fe_{NO} was measured at multiple expiratory flow-rates (50, 100, 200 and 250 ml/s). The gradient and intercept of a regression line between NO elimination (V'_{NO}) and flow-rate (V'_E) are recorded as Calv_{NO} (ppb) and J'aw_{NO} (pl/s), respectively.

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 Fe_{NO} 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, $p < 0.001$) (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. 2c and d). $\text{Fe}_{\text{NO}50}$ 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, $\text{Fe}_{\text{NO}50}$ 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, $\text{Fe}_{\text{NO}50}$ 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 v 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 FEV_1 , FVC or FEF_{25-75} . 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 Fe_{NO} measurements in allowing assessment of NO biosynthesis specifically from the bronchial and alveolar compartments of the lung.^{8,14,15,19–22} 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

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).

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

Data unavailable.

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 (Supplementary Table 1) we similarly found comparable alveolar NO levels. Paraskakis et al. also found comparable levels of alveolar NO between PCD and controls.¹⁴ 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.

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).

Nitric oxide parameter	Group			
	Healthy controls	PCD	Cystic fibrosis	Asthma
nNO (ppb) ^a	772 (690–886)	27 (16–76)**	501 (450–608)	769 (560–1124) ^c
FeNO ₅₀ (ppb)	16.7 (10.8)	8.8 (7.3) ^{b,d}	14.4 (11.2) ^b	43.4 (41.2) ^b
J'aw _{NO} (pl/s)	720 (514)	264 (209) ^{a,b,d}	519(495) ^b	2100 (1935) ^{a,b}
Calv _{NO} (ppb)	3.5 (1.3)	1.7 (0.8)** ^d	2.5 (1.2)*	5.4 (3.5)

^a Median (IQR).

^b Adjusted for weight.

^c $n = 15$ as 3 patients unable to perform nNO test.

^d $n = 13$ as 1 patient unable to perform FeNO tests.

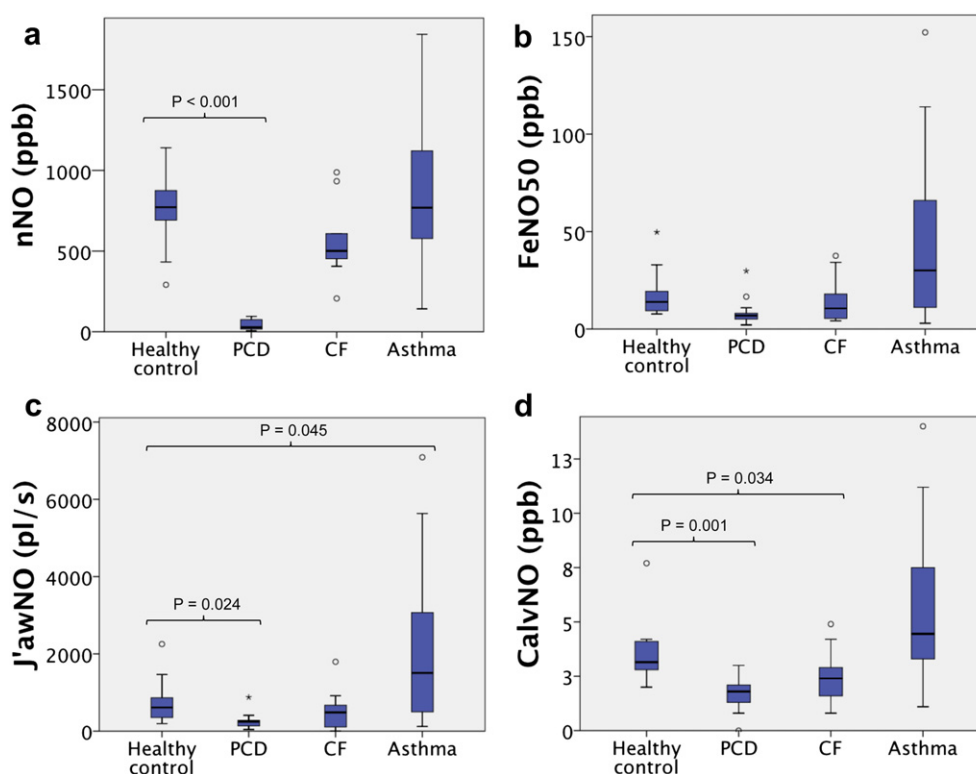


Figure 2 (a–d) - Box and whisker plot comparisons of (a) nasal nitric oxide concentration (nNO); (b) fractional exhaled nitric oxide concentration at 50 ml/s (FeNO_{50}); (c) bronchial nitric oxide flux (J'_{awNO}) and (d) alveolar nitric oxide concentration (CalvNO) in 18 healthy controls, 14 primary 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.^{7,20,22,24–26} It has generally been found that high FeNO levels are seen in asthma, particularly in under-treated asthmatics, and that this is reduced with ICS.²⁶ This has been demonstrated to originate from the bronchial region whilst alveolar NO remains relatively normal and this is consistent with our data.^{27,28} A recent Cochrane review concluded that FeNO -guided steroid therapy in asthma should not be recommended.²⁵ 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.^{19,24}

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.^{21,29} 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.^{21,29} 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).²¹ 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.³⁰ 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 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.^{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.^{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.

Fe_{NO} was measured according to ATS/ERS guidelines to ensure accuracy and repeatability of readings.¹⁷ Fe_{NO} is non-invasive, acceptable to patients and highly reproducible.¹⁷ Contamination of Fe_{NO} with nNO was avoided by exhalation against a minimum mouthpiece pressure of 5 cm H₂O.^{17,36} This process causes velum closure has been validated by nasal CO₂ measurement and nasal argon insufflation studies.^{36,37} However given that nNO levels are almost 40 times greater than Fe_{NO50} in our healthy children, even small amounts of contamination from the nasal cavity would lead to significant changes in Fe_{NO}. The NIOX[®] Flex analyser enables inhalation of NO-free air to control for the effect of ambient NO concentration. Furthermore a study in 1005 children found that ambient NO did not affect Fe_{NO} when measured according to ATS/ERS guidelines.³⁸ Similarly we found no relationship between ambient NO and NO parameters. Measures were taken to control for non-disease-related factors that may influence Fe_{NO} with groups being age and sexed matched.¹⁷

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.^{19,24} In CF the standard practice of monitoring spirometry in what is a disease of the peripheral airways is widely considered to be inadequate. While there is increasing literature on the benefit of lung clearance index in monitoring CF disease progression its availability in the clinical setting is presently limited.^{39–42} 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.

Acknowledgements

We would like to thank Helen Moyses (Biomedical Research Unit (BRU) Statistician for the NIHR Respiratory and Nutrition BRU, University Hospital Southampton) for her advice on appropriate statistical analysis for our study. The Primary Ciliary Dyskinesia Centre Southampton is funded by the National Specialised Commissioning Team. This study was supported by the Southampton NIHR Wellcome Trust Clinical Research Facility.

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>.

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Supplementary table 1 - Nitric oxide parameters in healthy adult controls (Data presented as mean (SD) unless stated)	
Nitric oxide Parameter	Healthy control adults
nNO (ppb)[#]	859 (546-1053)
Fe_{NO50} (ppb)	23.4 (22.4)
J'aw_{NO} (pl/s)	1078 (1031)
Calv_{NO} (ppb)	2.3 (1.5)
[#] Median (IQR)	