

NASAL NITRIC OXIDE AND NITRIC OXIDE SYNTHASE EXPRESSION IN PRIMARY CILIARY DYSKINESIA

Massimo Pifferi¹, Andrew Bush², Fabrizio Maggi³, Angela Michelucci⁴, Valentina Ricci³, Maria Elena Conidi⁴, Angela M. Cangiotti⁵, Alessandro Bodini⁶, Paolo Simi⁴, Pierantonio Macchia¹, and Attilio L. Boner⁶

¹Department of Pediatrics, University of Pisa, Italy

²Imperial School of Medicine at the National Heart and Lung Institute, London, UK

³Department of Experimental Pathology, University of Pisa

⁴Cytogenetics and Molecular Genetic Unit, University of Pisa, Italy

⁵Electron Microscopy Unit, Institute of Normal Human Morphology, Umberto I Hospital, Marche Polytechnic University, Ancona, Italy.

⁶Department of Pediatrics, University of Verona, Italy

Correspondence and requests for reprints should be addressed to:

Massimo Pifferi MD, PhD

Department of Pediatrics

University of Pisa

Via Roma 67 56126 Pisa - Italy

Fax: ++39 50 992641

Phone: ++39 50 992728

e-mail: m.pifferi@med.unipi.it

ABSTRACT

No study has evaluated the correlation between different expression of nitric oxide synthase (NOS) isoforms in nasal epithelial cells and nasal NO (nNO) level in primary ciliary dyskinesia (PCD).

Gene expression of endothelial (NOS 3) and inducible (NOS 2) NOS and their correlation with nNO, ciliary function and morphology were studied in patients with PCD or secondary ciliary dyskinesia (SCD). NOS 3 gene polymorphisms were studied in blood leukocytes.

Two hundred and twelve subjects were studied, (48 with PCD, 161 with SCD, 3 normal subjects). nNO correlated with mean ciliary beat frequency ($p=0.044$, $r=0.174$). The lower the nNO the higher was the percentage of immotile cilia ($p<0.001$, $r=-0.375$). A significant positive correlation between NOS 2 gene expression and nNO levels was demonstrated in all children ($p=0.001$, $r=0.428$), and this correlation was confirmed ($p=0.019$, $r=0.484$) in patients with PCD. NOS 2 gene expression was lower in PCD than in subjects with SCD ($p=0.04$). NOS 3 isoform correlated with missing central microtubules ($p=0.048$, $r=0.447$). nNO levels were higher in PCD with the NOS 3 894T mutation and this was associated with higher ciliary beat frequency ($p = 0.045$).

These results demonstrate a relationship between nNO, NOS mRNA expression, and ciliary beat frequency.

KEYWORDS: gene polymorphism, nasal nitric oxide, nitric oxide synthase, primary ciliary dyskinesia, secondary ciliary dyskinesia.

INTRODUCTION

Primary ciliary dyskinesia (PCD; MIM #242650) is a rare (1:15-30 000 live births) and usually autosomal recessive disease associated with situs inversus in nearly half the cases [1]. Impaired mucociliary clearance due to defective motility of cilia is the hallmark of the condition, but an additional sensitive, although not specific, feature of PCD is the very low level of nasal nitric oxide (NO). For this reason, nasal NO (nNO) has been proposed as a screening tool for PCD [1], even though the mechanism of low nNO in PCD is not known [2,3,4].

NO is a product of L-arginine metabolism by one of three isoforms of nitric oxide synthase (NOS): endothelial (NOS 3, located on chromosome 7), neuronal (NOS 1, located on chromosome 12) and inducible (NOS 2, located on chromosome 17). In general, At least in asthmatics, it is believed that NOS 2 is the major source of exhaled NO. NOS 2 is localized close to cilia in apical part of nasal epithelial cells [5]. It has been speculated that in PCD there is either uncoupling of the contractile process of the cilia from NOS leading to failure of NO production or that low nNO is an expression of a generalized disorder of airway NO metabolism with abnormalities in all three NOS isoforms [6].

It has been established that all three NOS isoforms may be regulated by gene induction at multiple levels, including transcriptional control [7]. The NOS 3 gene, which is located in chromosomal region 7q35–36, contains a number of variants, including a G/T polymorphism at position 894 in exon 7 [8]. This 894G/T substitution, which results in an aspartate rather than glutamate at position 298 in the NOS 3 protein, is of potential functional relevance since it was shown to correlate with severity of cardiopulmonary diseases in non-PCD patients [9], including asthma and cystic fibrosis [10, 11], but no data is available in PCD.

Since the low nNO in PCD may be related to altered expression of NOS isoforms, we hypothesised that in PCD there would be a reduction in NOS 2 and NOS 3 mRNA expression in the nasal mucosa, compared with a comparably infected and inflamed control group who do not have a congenital defect of ciliary function, namely children with secondary ciliary dyskinesia. The aim of

the study was to determine if NOS 2 and NOS 3 gene expression are different in nasal ciliated cells of children with PCD and secondary ciliary dyskinesia (SCD), respectively, and determine whether gene expression levels correlate with nNO. SCD patients had acquired ciliary abnormalities caused by viral or bacterial respiratory infections, and irritant injury of the mucosa produced by air pollution.

We studied NOS 3 since it is the only NOS isoform known to be localized in normal human nasal mucosa and is mainly associated with the ciliary basal microtubule membrane [5]. We assessed the relationship of NOS 3 gene expression to ultrastructural abnormalities of cilia. Furthermore, we analyzed the relationship of the 894G/T polymorphism to NOS 3 gene expression and nNO levels in PCD patients since it has been previously demonstrated that variants in the genes encoding for the nitric oxide synthases may act as disease modifier loci in cystic fibrosis [9]. Finally, we assessed if the polymorphism 894G/T in the NOS 3 gene was associated with particular ultrastructural and functional abnormalities of cilia.

MATERIALS AND METHODS

Subjects

Informed consent for all procedures was obtained from the children's parents and the Hospital Ethical Committee approved the protocol. Between January 2006 and June 2009, 212 children and adolescents (105 male, 107 female), aged 1 month to 17.5 years (median 6.2 IQR 5.1) with a clinical history and signs and symptoms suggestive of PCD were consecutively evaluated in Department of Pediatrics, University of Pisa, a National Referral centre. Investigations in all children included serum immunoglobulins, including total and specific IgE and IgG subclasses, lymphocytic subsets, sweat test and cystic fibrosis gene mutations. Young children were carefully observed during feeding to exclude swallowing problems and possible aspiration. Careful examination of the upper airway was performed to exclude clinical signs of supraesophageal complications of gastroesophageal reflux in all our patients. Sputum or a cough swab was collected for microbiologic analysis in subjects able to cooperate. In children too young to cooperate, a pharyngeal aspirate specimen was obtained after an overnight fast by a disposable catheter connected to a mucus extractor inserted into the mouth to a depth of 7 to 10 cm and drawn back while applying gentle suction.

Evaluations for PCD was performed when subjects were free from acute respiratory infection for at least 4 weeks and, in order to reduce the risk of infections causing secondary ciliary abnormalities, the patients were treated with oral antibiotics throughout this period. Patients were required to stop any pharmacological treatment except antibiotics in the previous 48 hours. Diagnosis of PCD and of SCD was made on the basis of structural and/or functional ciliary abnormalities. In all subjects, ciliary motion analysis, ultrastructural assessment of cilia on nasal brushings and nasal NO measurement was performed.

Nasal nitric oxide, nasal brush biopsy, ciliary motion analysis and ultrastructural studies were performed according to standard methodologies [12-16] detailed in the on-line supplement.

Nasal nitric oxide

Nasal nitric oxide evaluation was performed using standard methodology [10] which is also applicable to very young non-cooperative children [11]. Nasal air was sampled continuously with a constant transnasal flow of 1.8 mL/s for ≥ 30 s (CLD 88 Exhalyzer; EcoPhysics, Duernten, Switzerland).

Nasal brush biopsy

Samples, obtained from the inferior turbinate using a cytology brush (Microvasive, Milford, MA, USA), were suspended in 2 ml of Medium 199 fluid cell culture or in 2 ml of normal saline (2 samples), for immediate light-microscopic studies, transmission electron microscopy (TEM) evaluation, and NOS 2 and NOS 3 gene expression measurements. Ciliary motion analysis, ultrastructural assessment, and NOS 2 and NOS 3 mRNA measurements were performed by independent, blinded operators.

Ciliary motion analysis and ultrastructural studies

Samples for ciliary motion analysis were kept at 37°C and immediately transferred to a variable-thickness culture chamber. Ciliary morphology, motion pattern and beat frequency were quantitatively evaluated according to standardised methodology [1, 12]. Samples for ultrastructural studies were also prepared according to standardised methodology [13]. Cilia were studied by TEM at a final magnification of median 157,000 IQR 17,000. A mean of 14 ciliated cells and 120 transversely sectioned cilia were examined for each specimen. Quantitative analysis of ultrastructural alterations (expressed as per cent of observed abnormalities) was performed for the absence or shortening of dynein arms and for gross abnormalities in the central apparatus (central pairs/nexin links/radial spokes) [12]. A high proportion of these abnormalities, in association with specific motion patterns, a significant reduction in ciliary beat frequency, and a progressive worsening of respiratory disease were considered diagnostic of PCD. Other ciliary abnormalities were believed to be secondary to chronic inflammation and compatible with the diagnosis of SCD.

In atypical cases, the diagnosis of PCD was confirmed by ciliary activity evaluation after ciliogenesis in culture (14).

Measurement of NOS 2 and NOS 3 mRNA

NOS 2 and NOS 3 were evaluated in random samples of 71 and 53 children respectively (randomisation number generated by the program available online: <http://www.random.org/>). Nasal ciliated cells were immediately centrifuged and the cell pellets obtained were repeatedly washed in abundant phosphate-buffered saline (PBS), resuspended in 400 µl of PBS and finally stored at –80°C until use. Total RNA from ciliated cells extracted by the RNeasy Mini Kit (QIAGEN) was reverse transcribed to cDNA (Fermentas). NOS 2 and NOS 3 mRNA was measured by a commercial real-time quantitative PCR performed on ABI PRISM 7700 (Applied Biosystem) with primers and probes specific for each gene (TaqMan Gene Expression assays; Applied Biosystem). cDNA were also used as template for GAPDH quantification. Each sample was amplified in triplicate and a negative PCR control, without cDNA template, was included in each assay. The relative expression ratio of the target (NOS 2 and NOS 3 genes) was computed by the Relative Expression Software Tool [17] which allows for a relative quantification between groups and a normalization of the target genes with a reference gene. This software calculates the relative expression ratio (R) of a target gene on the basis on its real-time PCR efficiency (E) and the crossing point (Cp) difference (Δ) of an unknown sample versus a control called “calibrator”. In this study the “calibrator” was randomly selected by the principal investigator (MP) among three children with normal ciliary motion and normal transmission electron microscopy (TEM). The target gene expression is normalized by GAPDH, a non-regulated reference gene expression, using the formula is: $R = E_{\text{target}}^{\Delta C_p \text{ target (mean control-mean sample)}} / E_{\text{reference}}^{\Delta C_p \text{ reference (mean control-mean sample)}}$.

Genotyping

In 48 PCD children, blood was drawn to determine whether the NOS 3 gene polymorphism 894G/T was present. Genomic DNA was extracted using EZ1 DNA kit and BioRobot EZ1 (Qiagen) from peripheral blood leukocytes. The presence of the polymorphism 894G/T (rs1799983) in NOS 3 gene (Ensemble Gene ID: ENSG00000164867) was examined by sequencing analysis.

The primers used for amplification were the following: forward primer 5'-GGCTGGACCCCAGGAAAC-3' and reverse primer 5'-GCACCTCAAGGACCAGCTC-3'.

PCR conditions were: 5 minutes at 95°C, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds and a final elongation for 7 minutes at 72°C.

Purified PCR products were sequenced, with the same forward primer used for the amplification, according to the manufacturer's instructions using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

EcoPhysics, Duernten, Switzerland).

Statistical analyses

Baseline variables were expressed as median and interquartile range, and differences between medians were assessed by Mann-Whitney (Wilcoxon) W test. ROC curves were used to calculate the sensitivity and specificity of nNO for the diagnosis of PCD, and positive and negative predictive values were generated using the standard equations.

Correlations between quantitative results of ciliary motion analysis, ultrastructural evaluations, NOS 2 and NOS 3 gene expression measurements, presence or not of the polymorphism 894G/T (analyzed as a binary variable: GG = 0, GT or TT = 1), and nasal nitric oxide measurements were examined using Spearman's rank correlation test. The chi-squared test was used to evaluate the association between the presence of 894T variant in the NOS 3 gene and nNO levels ≥ 290 ppb in PCD patients and to assess the association between chronic airway infection with *Pseudomonas aeruginosa* and the presence of the 894T allele.

A p-value < 0.05 was considered statistically significant. All statistical calculations were performed using SPSS version 15.0 software for Windows (SPSS, Inc, Chicago, IL) for personal computers.

RESULTS

No child had CF (negative sweat test and negative mutation analysis), immunological abnormalities or, in young children, swallowing problems and clinical signs of gastroesophageal reflux. Ciliary motion analysis (abnormal motion patterns, including immotile cilia and/or very low ciliary beat frequency) and TEM evaluation of cilia (alterations of the central pair and dynein arms deficiencies, associated with a small proportion of swollen cilia and compound cilia) led to the diagnosis of PCD in 48 of 212 children (22.6%), 32 (66.7%) with situs inversus (Tab.1). Levels of nNO were < 290 ppb in 38 out 48 (79.2%) PCD subjects. In the remaining 10 patients with levels of nNO \geq 290 ppb, the diagnosis of PCD was confirmed by ciliary activity evaluation after ciliogenesis in culture. In our study group the NO cut-off with the best combination of sensitivity (74%) and specificity (80%) was 290 ppb which had a positive and negative predictive value of 76% and 78% respectively. Ciliary motion analysis demonstrated abnormal patterns in a small proportion of cilia, with some thick cilia and low ciliary beat frequency, compatible with the diagnosis of SCD in 161 patients (75.9%). In these subjects, TEM showed nonspecific abnormalities compatible with chronic inflammation (prevalence of swollen cilia and compound cilia). Three subjects had no abnormalities in TEM and ciliary function, one of whom was randomly selected as a healthy control for NOS 2 and NOS 3 gene expression measurements.

Significant correlations were found between ciliary motion analysis and TEM results. In particular, taking all 212 subjects together, abnormal motion patterns (as percent of motile cilia) was directly correlated ($p=0.04$, $r=0.121$) and mean ciliary beat frequency (immotile cilia excluded) was inversely correlated ($p<0.001$, $r=-0.346$) with percentage of pathologic cilia observed with TEM. Mean ciliary beat frequency was directly correlated and immotile cilia inversely correlated with

normal ciliary axoneme at ultrastructural evaluation ($p < 0.001$, $r = 0.346$ and $p < 0.001$, $r = -0.436$, respectively).

Considering PCD and SCD children together, nNO was very weakly correlated with mean ciliary beat frequency ($p = 0.044$, $r = 0.174$) (Fig.1) and inversely correlated with percent of immotile cilia on light-microscopy ($p < 0.001$, $r = -0.375$) (Fig.2).

Of the 71 patients in whom NOS 2 was evaluated 27 had PCD and 44 SCD. NOS 3 was evaluated in 53 patients (27 PCD, 26 SCD). The relative NOS 2 gene expression in nasal ciliated cells of children with PCD (Median:0.9 [IQR:1.9] fold increase *vs* normal control) was significantly lower ($p = 0.03$) than in subjects with SCD (Median:1.9 [IQR:3.5] fold increase *vs* normal calibrator), although there was considerable overlap; i.e., SCD children had greater NOS 2 gene expression than PCD or the normal control, which last two were not significantly different. There was no difference in NOS 3 gene expression between children with PCD (Median: 4.5 [IQR:18.3] fold increase compared with normal control) and subjects with SCD (Median: 7.4 [IQR:15.8] fold increase compared with normal control). There was a significant correlation ($p = 0.019$, $r = 0.484$) between NOS 2 gene expression and nNO in the 27 children with PCD, but not in SCD. However the significance is lost if the three outliers are excluded. In any case a significant positive correlation between NOS 2 gene expression and nNO levels was demonstrated in all children (PCD plus SCD) ($p = 0.001$ $r = 0.428$).

There was no correlation between the relative NOS 3 gene expression and nNO levels in any group. However, since NOS 3 isoform is localized at the basal membrane of ciliary microtubules, we sought and found a significant correlation with ultrastructural alterations such as missing central microtubules in patients with PCD ($p = 0.048$, $r = 0.447$), but not in subjects with SCD. This correlation disappears excluding the extreme outlier with more than 250% NOS 1 gene expression in relation to the healthy normal comparator. However, the numbers are small, and there is a wide scatter about the line of best fit.

The NOS 3 genotype was homozygous for the wild-type 894G allele in 22 patients; the T allele (n=26) was heterozygous (894G/894T) in 22 and homozygous (894T/894T) in 4. Median (IQR) nNO in G/G wild-type subjects was not significantly different from that in children carrying the T allele [93.0 ppb (132.3) vs 75.9 ppb (326.9)], however the numbers are small.

nNO levels \geq 290 ppb were observed in 10 PCD patients: 7 (5 adolescent females) with 894T mutant allele (26.9% of the total with T allele) and 3 patients homozygous for the 894G wild-type allele (13.6% of the total homozygous for the G allele).

No significant difference was found in NOS 3 mRNA between PCD patients homozygous for the 894G allele and those carrying the 894T allele in either the heterozygous or homozygous form with Median: 4.6 [IQR:17.7] and Median: 3.2 [IQR:7.5] fold increase vs normal calibrator, respectively.

Finally, the T allele (either in the heterozygous or homozygous) inversely correlated with the most typical ultrastructural alterations such as missing inner and outer dynein arms ($p=0.002$, $r=0.480$), and this was not the case with the wild-type G/G allele. In addition, the inner and outer dynein arms defect was significantly lower when 894T allele was present (Median in genotype 894G/894G versus 894G/894T plus 894T/894T was 47.5% [IQR 59.3] and 0% [IQR 2,6], respectively; $p=0.006$). There was a significant difference in ciliary beat frequency between PCD patients carrying the T variants and PCD patients homozygous for the G allele ($p = 0.0459$).

Moreover, chronic infection with *Pseudomonas aeruginosa* was found in 7 out of 22 (31.8%) of the patients homozygous for the G allele and in 5 out of 26 (19.2%) patients carrying the T variants ($p=0.5$). However 3 out of 6 pubertal female patients homozygous for the G allele were chronically infected with *Pseudomonas aeruginosa*, compared with none of 5 pubertal female patients carrying the T alleles, but again the numbers are small and any conclusions must be provisional pending confirmation.

DISCUSSION

The main findings of this study are that NOS 2 mRNA levels correlate with nNO in a large group of patients with PCD and SCD when taken together; this correlation is driven essentially by PCD subjects; and levels of nNO correlate with ciliary functional abnormalities. Furthermore, exploratory analyses have suggested that NOS 3 polymorphisms may associate with particular dynein arm defects, although this is a hypothesis generating observation which requires confirmation in a larger group of patients. The strengths of the study include the careful categorisation of a large number of patients with state of the art diagnostic tests in a single centre.

There are a number of factors to consider when interpreting these data. Firstly, the sensitivity and specificity of nNO measurements in this group appears to be different from that reported elsewhere [2]. This may relate to different techniques or equipment, and it is important for each centre to establish their own normal ranges [18]. We confirmed the diagnosis of PCD by tissue culture in all patients with higher than expected nNO [16], so we are confident that the diagnosis is correct. Secondly, we could only study NOS 2 and NOS 3 mRNA, not protein, so we cannot exclude the possibility that post-translational modification may have affected our results. Finally, our numbers are relatively small, especially for genetic analyses. This is to some extent inevitable in a condition as rare as PCD, but means that in particular the genetic work needs confirmation in a second cohort of patients.

Ciliated epithelial cells produce NO which is a regulator of CBF throughout NO-cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) pathways [19]. There is good evidence that cAMP and cGMP regulate mammalian CBF via the activation of axonemal protein kinase A and protein kinase G to phosphorylate a dynein light chain, which in turn possibly mediates a switch from the slow to the fast dynein-duty cycle and thus increases CBF [20]. Low levels of nNO correlate with impaired mucociliary function in the upper airways [21]. Endogenous NO, principally synthesized by NOS 2 within the respiratory epithelium of the nasal sinuses, has been demonstrated to increase baseline CBF of airway epithelia both *in vitro* [22, 23] and in an

animal model [24]. Here we extend these observations by showing that there is a correlation between nNO and ciliary motion in children with SCD or PCD. An increased ciliary beat frequency was correlated with the level of nNO (figure 1) and a higher percentage of immotile cilia was correlated with low nNO (figure 2). It has to be acknowledged that the scatter is wide; nonetheless the observation has been made in a large number of patients. In human studies, instillation of the NO donor sodium nitroprusside into the nose of six volunteers was found to increase nasal mucociliary activity [25] and the administration of L-arginine was shown to improve ciliary beat frequency in PCD patients [26]. This body of evidence suggests that reduced nNO, as well as being a marker for PCD, may also contribute to the pathophysiology, and we speculate that NOS polymorphisms may be modifier genes for the PCD clinical phenotype. However, multicentre prospective studies are needed to confirm this.

There is no obvious reason for the decreased nNO in PCD patients. All three NOS isoforms have been found in airway epithelial cells [5, 27, 28] and here we have shown that NOS 2 and NOS 3 are found in nasal epithelial cells of PCD patients. The lower NOS 2 mRNA levels in PCD compared to SCD together with the correlation between nasal NO and NOS 2 in PCD children suggests that impaired NOS 2 function may significantly contribute to the reduced nasal NO levels in PCD subjects. However, further work is needed to confirm this speculation. NO is an important host defence molecule, and is important in the response to infection and inflammation. However, the relative NOS 2 gene expression was reduced in PCD compared to subjects with SCD despite potential greater infectious stimuli in the former group. Thus it is tempting to speculate that in PCD there is a reduced potential for NOS 2 expression and production as previously hypothesised [6]. However, we did not measure the burden of nasal infection and inflammation and the effect of antibiotic treatment, so further work is clearly needed before any conclusions can be drawn as to the dose relationship between infection, inflammation and NOS 2 expression in these two groups.

As suggested by the same group [6] no significant different gene expression for NOS 3 was observed between PCD and SCD patients, confirming that in PCD there is no generalised disorder

of NO handling. It may be that the contribution of NOS 3 to the production of NO in the lung periphery is important but it does not seem to play a major role in the nose where NO production seems mainly driven by NOS 2. No correlation was found in any patient group between NOS 3 gene expression and nasal NO. Nevertheless, in preliminary exploratory work, NOS 3 gene expression significantly correlated with the percentage of missing central microtubules only in PCD patients and in relation to the ultrastructurally localization of NOS 3 near the basal body of the microtubules of the cilia [5] this may be a causally linked effect. However, further work is needed to confirm whether this is a real finding, and if so, the significance of this observation.

In our PCD patients, NOS 3 polymorphisms did not influence median nNO but in 70% of patients with nNO > 290 ppb the 894T allele was present. The allele (heterozygous or homozygous) inversely correlated with the most typical ultrastructural alterations such as missing inner and outer dynein arms ($p < 0.001$, $r = -0.563$), and this was not the case with the wild-type G/G allele. In fact, the prevalence of inner and outer dynein arm defects was significantly lower when the 894T allele was present ($M \pm SD$ in genotype 894G/894G versus 894G/894T plus 894T/894T $36.8 \pm 30.9\%$ and $7.5 \pm 16.1\%$, respectively; $p = 0.0003$). Clearly there are numerous dynein arm genes, and linkage disequilibrium between NOS 3 and dynein arm genes is thus an unlikely explanation for this finding. However, it may be that in some as yet unknown way, NOS 3 polymorphisms modify the expression of dynein arm genes, leading to more or less dynein arms for a given gene defect. Again, this finding is preliminary and requires confirmation by other investigators.

Thus, around 25% of the total patients with the T allele had higher than expected nNO ppb. It has been observed in CF that NO levels were significantly higher in females with an 894T mutant allele, compared with female patients homozygous for the 894G wild-type allele and that chronic infection of airways with *Pseudomonas aeruginosa* was significantly less frequent when carrying an 894T mutant allele as compared with wild type [11], but this is the first report of these findings in PCD. In our study population, chronic infection with *Pseudomonas aeruginosa* was found in 7 out of 22 (31.8%) of the patients homozygous for the G allele and in 5 out of 26 (19.2%) patients

carrying the T variants. None of the five post-pubertal females with the T allele was positive for *Pseudomonas aeruginosa* and all the sputum culture positive patients were male. The apparent protective effect of the T allele on airway infection was observed also in our study in post pubertal female patients and we speculate that this could be related to circulating estrogen, which activates plasma membrane-associated estrogen receptors coupled to NOS 3 [29]. However, we do not have any estrogen measurements in these patients.

In conclusion we suggest that NO is implicated in the modulation of ciliary function in PCD patients. NOS 2 but not NOS 3 gene expression is lower in PCD patients and NOS 3 gene expression may be correlated with loss of central microtubules. This reflects the correlation between NO and ciliary function, and may explain some of the clinical variability of the disease.

ACKNOWLEDGEMENTS

We are grateful to Fondazione Carlo Laviosa for financial support.

TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION

Study population n. (%)	nasal NO n. (%)		NOS 3 mRNA (fivnc) ¹ Median (IQR)	NOS 2 mRNA (fivnc) ¹ Median (IQR)	NOS 3 gene polymorphisms	
	<290 ppb	≥290 ppb			T/T G/T n. (%)	G/G n. (%)
PCD 48 (22.6)	38 (79.2)	10 (20.8)	4.5 (18.3) ²	0.9 (1.9) ⁴	4 (8.3) 22 (45.8)	22 (45.8)
SCD 161 (75.9)	21 (13)	140 (87)	7.4 (15.8) ³	1.9 (3.5) ⁵	–	–

¹ fivnc: fold increase vs normal control.

² in 27 PCD patients.

³ in 26 SCD patients.

⁴ in 27 PCD patients.

⁵ in 44 SCD patients.

FIGURE LEGENDS

Figure 1: Correlations between nasal NO and mean ciliary beat frequency ($p=0.044$, $r=0.174$).

Figure 2: Correlations between nasal NO and % of immotile cilia at light-microscopy ($p<0.001$, $r=-0.375$).

Figure 3: Correlation between the relative NOS 2 gene expression and nasal NO levels (all patients together: $p=0.03$, $r=0.278$; only PCD patients: $p=0.008$, $r=0.538$). Statistical significance is lost if the three outliers (marked with arrows) are excluded.

Figure 4: Correlation between NOS 3 isoform gene expression and ultrastructural alterations such as missing central microtubules in patients with PCD ($p<0.001$, $r=0.735$).

Figure 5: Percent of missing inner and outer dynein arms in patients with the wild-type G/G allele and in those heterozygous or homozygous for the T allele of NOS 3 ($p=0.0003$). Horizontal lines represent means.

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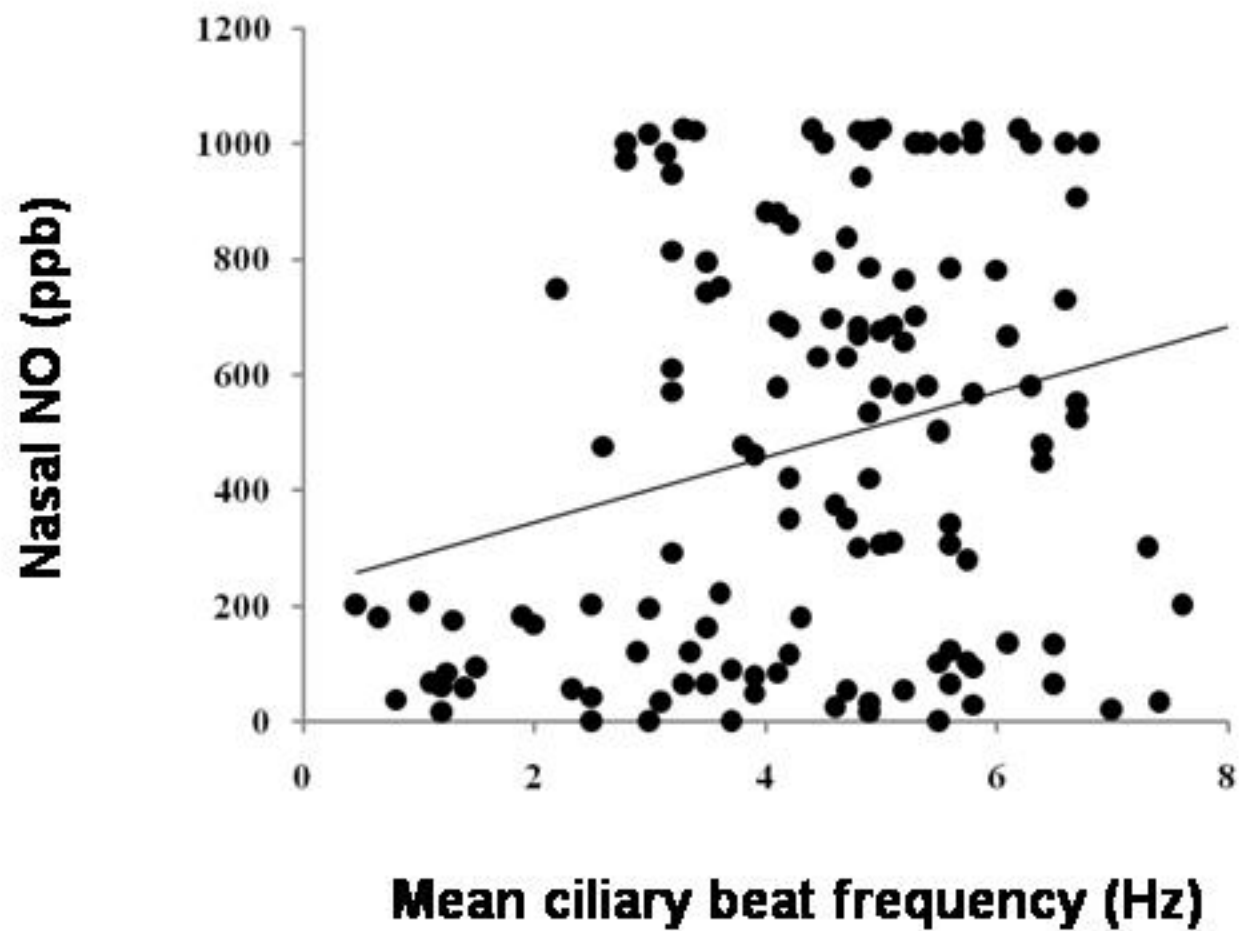


Fig. 1

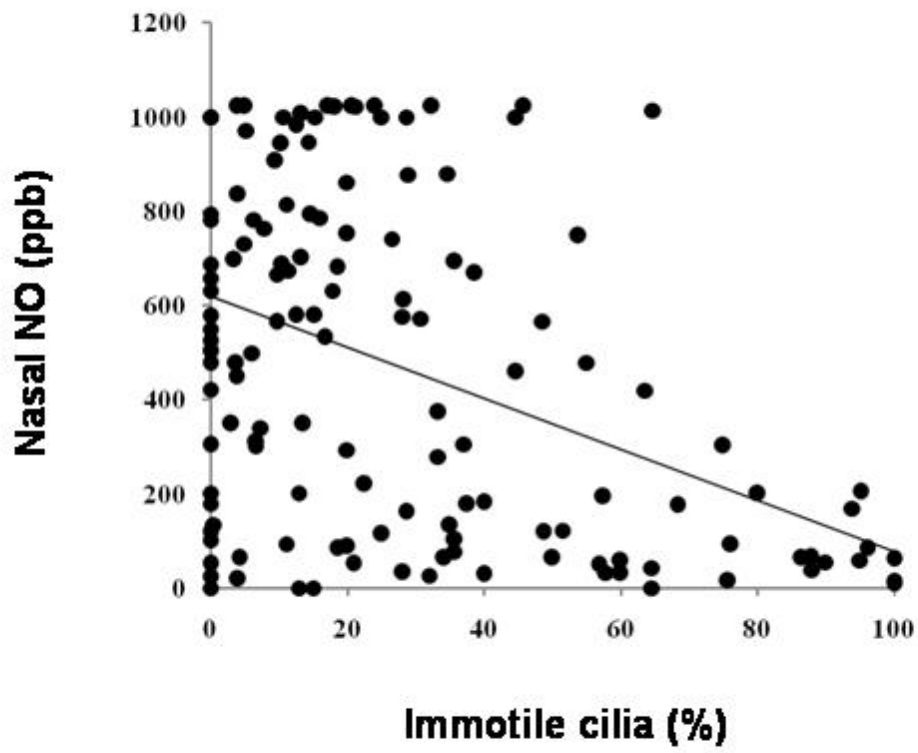


Fig. 2