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Nasal nitric oxide for early diagnosis of primary ciliary dyskinesia: Practical issues in children

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Summary
Background: Primary ciliary dyskinesia (PCD) is a genetic disease characterized by abnormally beating cilia. In these patients, levels of nasal nitric oxide (nNO) are lower than those observed in healthy subjects.
Objectives: We identify the nNO levels in healthy pre-school uncooperative children and in PCD patients, in order the application of nNO measurement in the early identification of young children with PCD.
Methods: We measured nNO in 77 healthy children (50 uncooperative and 27 cooperative) and in 10 PCD patients. Fifteen cooperative healthy children were also asked to perform an uncooperative test.
Results: PCD patients presented low nNO levels (29.7 ± 5.7 ppb) compared to those observed in healthy children (358.8 ± 35.2 ppb; $p < 0.05$). nNO levels were increased in healthy cooperative children (650 ± 60.6 ppb; $p < 0.05$) as compared to those uncooperative aging more than 6 month (309.1 ± 45.9 ppb; $p < 0.05$) or less (128.1 ± 16.2 ppb; $p < 0.05$). Twenty-four uncooperative children with nNO values $\leqslant 200$ ppb performed a second evaluation at least 6 months later and mean levels increased from 104.7 ± 10.5 ppb to 169.9 ± 19.6 ppb ($p < 0.05$). In the 15 collaborative children nNO levels were higher during the breath holding manoeuvre (687.7 ± 96.9 ppb) than during the tidal breathing manoeuvre (335.9 ± 57.9 ppb; $p < 0.05$).
Conclusions: Healthy children have higher nNO levels than PCD patients. In 15% of uncooperative healthy children can be found low nNO levels, similar to PCD patients, but those values increased some months later, in successive evaluations. Nasal NO may be used for PCD screening even though repeated evaluations may be necessary in young children.

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Introduction

Primary ciliary dyskinesia (PCD) is a genetic disease characterized by defective motility of cilia, in most cases related to an ultrastructural defect,[1] which results in impaired mucociliary clearance of the upper and lower airways[2] associated with recurrent-chronic respiratory symptoms.[1,3]

Since respiratory symptoms are also common in healthy children and the disease is relatively rare (1 in 15–30,000 live births, in white population), the diagnosis of PCD is often missed for a long time.[4] Furthermore, diagnostic investigations such as the demonstration of ultrastructural defect at transmission electron microscopy and ciliary motion analysis require both expertise and laboratory facilities which are not widely available.

In order to prevent lung function deterioration due to inadequate treatment, an early identification of PCD patients is warranted.[5,6] Since nNO level in PCD patients has been observed to be 80–90% lower than in healthy controls,[7–15] including affected infants,[16,17] this parameter could provide useful first-line information in the diagnostic algorithm of PCD. Nevertheless, while normal values are available for school aged children,[18] reference values are lacking in pre-school aged children and infants who cannot perform the nNO test according to ERS/ATS guideline.[19]

The aim of this study was to identify the nNO levels in healthy infants and pre-school children devoid of signs and symptoms suggestive of ciliary dyskinesia (Table 1). A particular regard was dedicated to methodological issues in these uncooperative subjects, in order to better address the potential application of nNO measurement in the early identification of young children with PCD.

Table 1 Signs and symptoms suggestive of PCD.[2,3]

Neonatal period:

- respiratory distress or pneumonia in term neonates with no obvious predisposing cause
- rhinitis and/or nasal congestion that remain constant over time
- situs inversus
- moist sounding cough is unusual in this period but suggestive
- complex congenital heart disease, esophageal and biliary atresia, hydrocephalus
- positive family history of PCD

Infant and older child:

- chronic cough with sputum production
- rhinosinusitis
- chronic secretory otitis media with prolonged otorrhoea after tympanostomy
- pneumonia
- bronchiectasis
- repeated courses of antibiotics for chest infections
- atypical asthma refractory to treatment

Adults:

- as for older children with reduced importance of otitis media
- subfertility or infertility in male
- ectopic pregnancy

Patients and methods

Subjects

A total of 87 subjects participated to the study. Of these, 10 patients (7 males, mean 17 years) with PCD, two of whom were uncooperative children, diagnosed by electron microscopy[2,20] served as positive controls. Main clinical features for these patients are summarized in Table 2. Seventy-seven subjects were healthy children (46 males, 31 females), 50 of which were uncooperative infants aged less than 6 months ($n = 26$) or between 6 and 12 months ($n = 24$) and 27 were school-aged children (mean 7 years) able to perform the test procedures according to guidelines.[19]

The subjects were selected among those who had never received inhaled corticosteroids or nasal decongestant drugs and did not have adeno- or tonsillectomy.

The measurements were part of routine clinical evaluation.

Nasal NO measurements

Exhaled nasal nitric oxide (nNO) level was measured by inserting a nNO-inert olive in one nostril, completely occluding the nostril to avoid ambient air sampling.[18,19] The controlateral nostril was left open. The olive was connected *via* a Teflon® tube at the analyser and the nasal air was sampled continuously with a constant transnasal aspiration flow of 300 mL min^{-1}.[19,21] The nNO

Table 2 PCD patients' characteristics.

Patients no.	*Situs inversus (yes/no)*	Bronchiectasis (yes/no)	Sinusitis (yes/no)	Cilia ultrastructural defect
1; c	N	Y	Y	I-ODA
2; c	N	Y	N	I-ODA
3; u	Y	N	N	IDA
4; c	N	Y	N	I-ODA
5; c	Y	Y	N	I-ODA
6; c	N	Y	N	IDA
7; c	N	Y	N	ODA
8; c	N	Y	N	I-ODA
9; c	Y	Y	Y	I-ODA
10; u	Y	N	N	I-ODA

c, cooperatine; u, uncooperative; I-ODA, inner and/or outer dynein arms.

was measured "online" with a NIOX chemiluminescence analyser (Aerocrine, Stockolm, Sweden), which was calibrated at least every 14 days using certified calibration gas (NO, 1460 ppb). The nNO signal was sent to a computer data acquisition program (NIOX, nasal mode; Aerocrine) that displayed real-time measurements.[18]

Cooperative children were asked to take a deep breath and hold it for 10 s while the average nNO concentration was calculated at the plateau between 7 and 10 s after breath-hold according to ERS and ATS guidelines.[19] Moreover, 15 of these cooperative subjects were asked to perform an uncooperative test mimicking the condition of infants, i.e. the nasal sampling was performed continuously for 30 s during tidal breathing as it was done for the uncooperative infant group. For all subjects the manoeuvre was performed in triplicate. Measurement of ambient NO concentration was recorded every day.

Statistical analysis

Data are presented as mean ± standard error of the mean ($X \pm$ SEM). Between groups comparisons were performed by ANOVA for multiple sample comparison. Paired *t*-test was used for the comparison within the group of cooperative children when performing the nNO measurement according to the guidelines as compared to mimicking the condition of infants. A p value of <0.05 was considered significant.

The accuracy of the test was calculated according to the following formulas:

- Sensitivity (Se) = True positives/(true positives+false positives) × 100.
- Specificity (Sp) = True negatives/(true negatives+false positives) × 100.

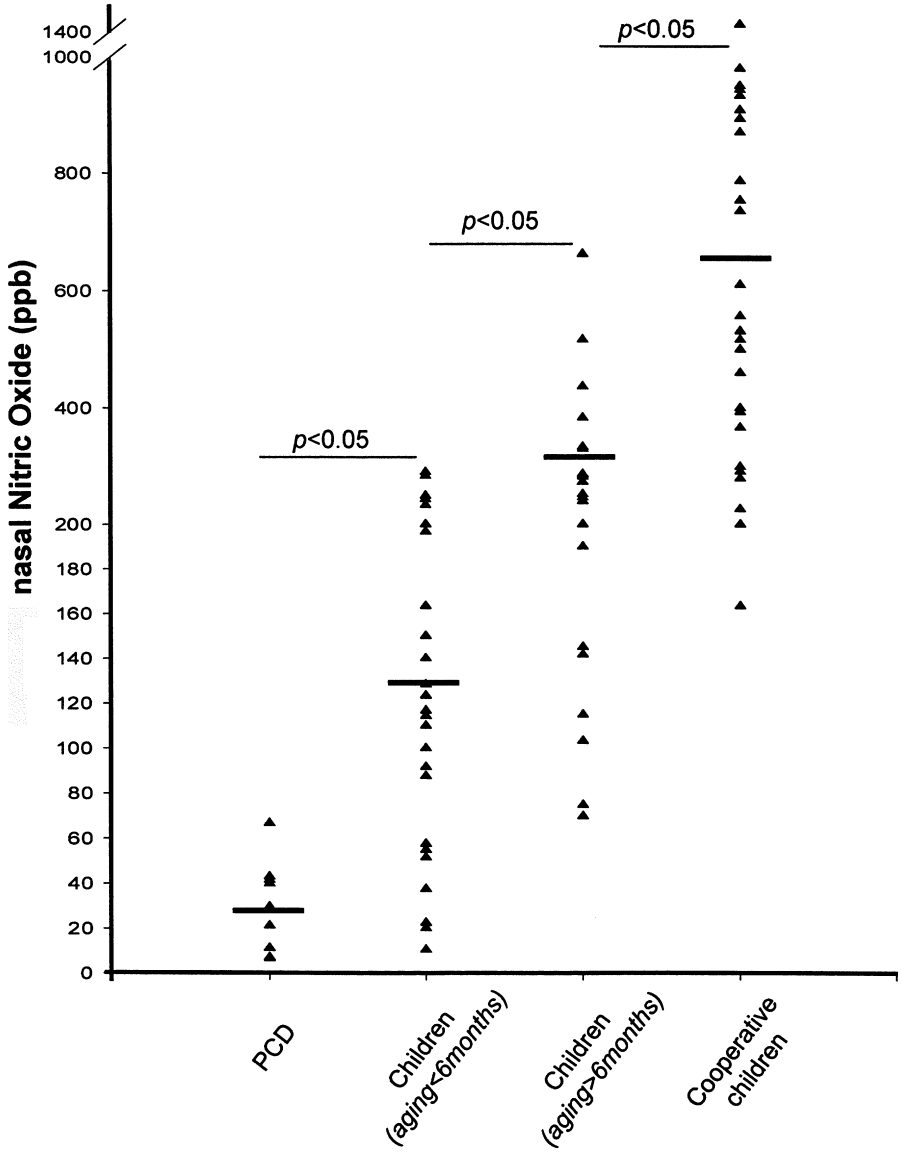

Figure 1 Individual values of exhaled nNO levels in PCD patients, in healthy children aging more or less than 6 months and in cooperative school-aged children. Horizontal lines represent mean value.

- Positive predictive value (PPV) = True positives/(true positives+false positives) × 100.
- Negative predictive value (NPV) = True negatives/(false positives+True negatives) × 100.
- Receiver operating characteristic (ROC) curves for eNO were constructed.

Results

nNO measurements were performed in all the children and no adverse event was observed. When healthy subjects were considered altogether ($n = 77$), independently from the age and from their ability to perform the test according to guidelines (methods A or B), nNO values ranged from 10.6 to 1456 ppb (mean 358.8 ± 35.2 ppb). In the 10 subjects with PCD the nasal NO levels range was from 6.7 to 66.7 ppb with a mean values of 29.7 ± 5.9 ppb, being significantly lower than the mean value of the whole group of all healthy children ($p < 0.005$). The two infants with PCD had, respectively, nNO values of 7.2 and 41.6 ppb. All individual data measured in children are presented in Figure 1.

Considering the whole group, ROC curves indicated that, in our study group, a nNO value of 68 ppb had the best combination of sensitivity and specificity for predicting the diagnosis of PCD (Se = 100%, Sp = 90%) (Figure 2, panel A). The PPV and NPV values of nNO of 68 ppb were 56% and 100%, respectively.

When the healthy population was analysed by ANOVA comparing the two subgroups of uncooperative children and the group of cooperative school aged subjects, a significant difference among groups was observed ($p < 0.01$). The subgroup of cooperative school aged children ($n = 26$) had a mean nNO level of 650 ± 60.6 ppb, which was significantly higher than in uncooperative healthy children either aging more than 6 months (309.1 ± 45.9 ppb, $p < 0.05$) or less than 6 months (128.1 ± 16.2 ppb, $p < 0.05$).

Considering the group of cooperative healthy children the ROC curves indicated that, in our study population, a nNO value of 71 had a combination of Se = 100% and SP = 100% for excluding the diagnosis of PCD (Figure 2, panel B). As a consequence, the PPV and NPV values for nNO cutoff of 71 ppb were both 100%. Excluding the uncooperative children from the group of PCD patients ($n = 2$) and considering the cooperative PCD children ($n = 8$) as compared to all the healthy children, ROC maintained the same characteristics.

In the whole group of uncooperative children below the age of 12 months, 4 out of the 50 (8%) showed nNO values < 50 ppb (with cut-off value 50 ppb: Se = 90%, Sp = 92%, PV+ = 69%, PV− = 98%) while 12 (24%) had nNO values < 100 ppb (with cut-off value 100 ppb Se = 100%, Sp = 76%, PV+ = 45%, PV− = 100%).

Also in this group, ROC curves indicated that a nNO value of 68 had the best combination of sensitivity and specificity for excluding the diagnosis of PCD (Se = 100%, Sp = 84%). The PPV and NPV values for nNO cutoff of 68 ppb were 56% and 100%, respectively. No significant difference was shown in the comparison of the ROC curve from this group versus the one from cooperative children.

As significant difference in nNO levels was also observed between uncooperative children aged less than 6 months vs more those aged 6 to 12 months ($p < 0.05$), a separate analysis to assess the accuracy of test in the youngest group was performed. In this group, the percentage of false positives was higher, being 15% (4 out 26) with cut-off value of 50 ppb (Se = 90%, Sp = 85%, PV+ = 69%, PV− = 96%) and 38% (10 out of 26) with cut-off value of 100 ppb (Se = 100, Sp = 61%, PV+ = 53%, PV− = 100%). In this subgroup, ROC curves indicate that a nNO value of 54 ppb had the best combination of sensitivity and specificity for predicting the diagnosis of PCD (Se = 90%, Sp = 81%) (Figure 2, panel C). The PPV and NPV values of nNO cutoff of 54 ppb were 64% and 95%, respectively. A comparison of the ROC curve obtained considering all the cooperative children versus the uncooperative aged below 6 months showed a significant difference (AUC 1 vs 0.89; Kendal-Tau = 0; $Z = 2.045$).

In order to assess a possible effect of the age in the uncooperative healthy children, 24 of the 28 with nNO lower than 200 ppb were re-evaluated after 6–8 months from the first measurement. In these subjects, nNO significantly increased from 104.7 ± 10.5 ppb to 169.9 ± 19.6 ppb ($p < 0.05$) (Figure 3). The same re-evaluation after 6–8 months was performed also in the two non-cooperative children with PCD. For these patients the nNO values were 41.6 and 7.2 ppb at the first measurement. At the second

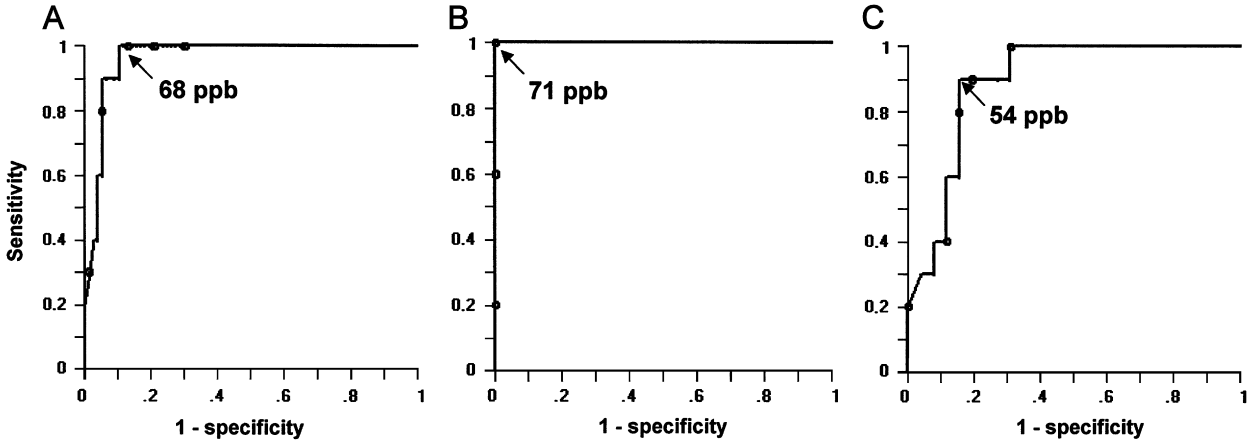

Figure 2 ROC curves for nNO for PCD patients and the whole group of healthy controls (panel A), cooperative healthy controls (panel B) and healthy infants below 6 months of age (panel C).

test the value practically did not change being 40.0 and 10 ppb, respectively.

In the 15 cooperative children who performed both the standardized manoeuvre with 10 s of breath holding and the manoeuvre without breath holding, mimicking the condition of younger subjects, nNO levels were (687.7 ± 96.9 ppb and 335.9 ± 57.9 ppb, respectively in the two different experimental conditions ($p < 0.05$) (Figure 4). No healthy school-aged children had nNO values less than 150 ppb when performing the test as recommended by guidelines[18] but 2 of the 15 (13.3%) children had values lower than 100 ppb during the uncooperative manoeuvre.

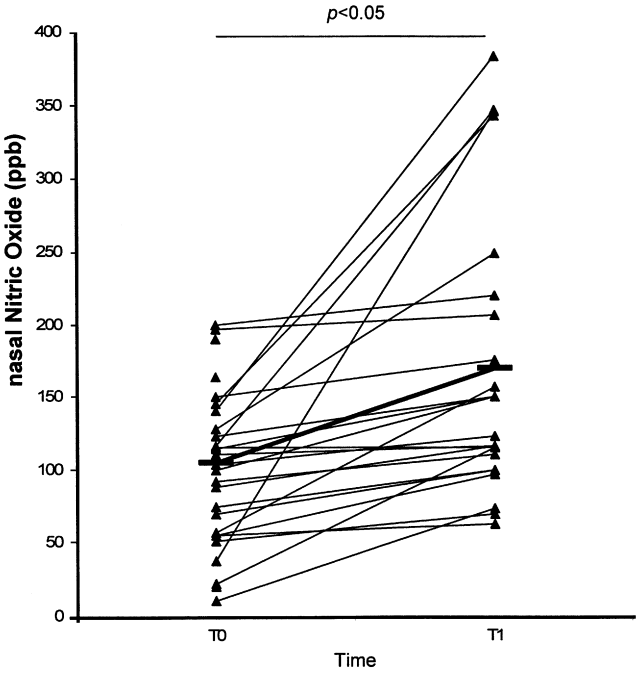

Figure 3 Individual values of nNO in young uncooperative children performing two evaluations at least 6 months apart: T0, first evaluation and T1, second evaluation. Horizontal lines represent mean value.

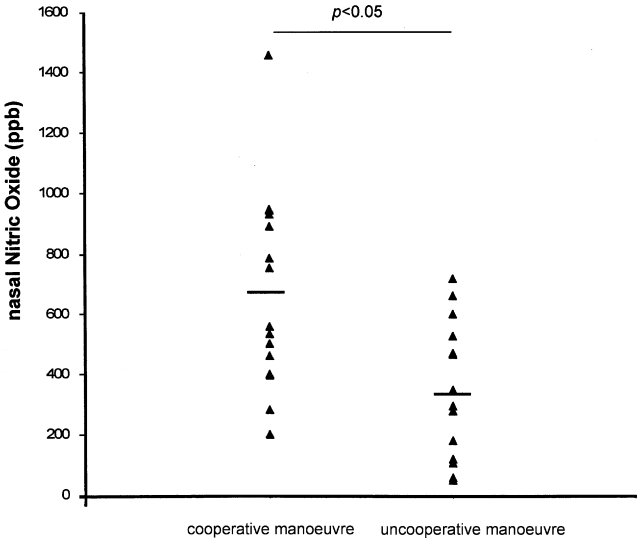

Figure 4 Individual values of exhaled nNO levels in healthy cooperative children during the cooperative and uncooperative manoeuvre. Horizontal lines represent mean value.

Discussion

NO is synthesized by epithelial ciliated cells of the nasal cavity and paranasal sinuses where, because of its high concentration of several hundreds parts per million,[4] it can exert antibacterial and antiviral effects. This could be one of the main mechanisms contributing to ensure sinus sterility in healthy subjects. Furthermore, there is growing evidence that NO plays a crucial role in the regulation of ciliary airway motility[22] and several studies have shown lower levels of nNO in children with PCD as compared to healthy age-matched subjects.[7–15] Recently, low nNO have also been reported in infants with PCD[16,17] and in atypical PCD patients with persistent abnormal ciliary motility but lacking the classical ultrastructural defect.[23] The body of these recent reports reinforce the suggestion that nNO can be used a screening tool for the early identification of PCD patients, thus reducing the necessity to perform electron microscope or kinetic evaluation in a wide number of patients for which the suspect of PCD could be reshuffled by normal levels of nNO. Nevertheless, in presence of persisting symptoms PCD needs to be ruled out with the above-mentioned validated tools.

The results of the present study confirm that the mean level of nNO in school-aged children is similar to those previously reported[18] despite the use of diverse sampling flows, respectively, 300 and 700 mL min^{-1}. Different sampling flows have been used by different authors (Table 3) and somehow complicates between-study comparison. Since lower nNO ppb levels are obtained with higher aspiration flows,[24] a standardization of sampling flow is warranted in order to address a threshold for normal values. Even with the same operating procedures, including sampling flow, the results of our study show that in children below the age of 12 months the mean nNO levels is significantly different from cooperative subjects, with some values overlapping those of PCD patients. This is particularly true for the group of children younger than 6 months, who presented a mean nNO level even lower than that observed in the subjects aged 6 to 12 months. Furthermore, all of the eight uncooperative subjects with nNO values overlapping those of PCD patients were in the group aged less than 6 months. This observation is in agreement with previous ones which documented that nasal NO excretion is considerably reduced in infants compared to older children and adults and that it increases both with increasing body weight[25] and with postconceptional age.[26,27]

Lower levels of nNO in younger children can be explained by the fact that paranasal sinuses in infants are only partially pneumatised with less sinus surface than older children and therefore NO supply to the nasal cavity might be lower than in older subjects. Furthermore, in our study, low nNO levels were also observed when school-aged cooperative children performed the test in an uncooperative fashion, continuing tidal breathing without breath-holding during sample collection. Since during tidal breathing the

Table 3 Nasal NO output reported by several authors.

Author, year (Ref.)	Sample flow (mL min^{-1})	Mean nasal NO values (ppb)		Mean age (years)
		PDC patients	Healthy	
Baraldi, 2004[16] †	110	100	295	0.4
Karadag, 1999[7]	240	97	664	10.8
Naragan, 2002[9]	250	60.3	716	10.8
Horvath, 2004[10]	250	54.5	663	34
Lundberg, 1994[8]	800	4	221	7.7
Corbelli, 2004[13]	1200	13.7	223.7	11.4
Stehling, 2006[17] †	–*	<5	171.1	0.08
Wodehouse, 2003[11]	–*	65.7	759.1	34.2
Grasemann, 1999[15]	–*	72.1	1195	23

*Sample flow not declared.
†Case report.

nasal concentration of NO can be diluted by the air coming from lower airways, which contains much lower concentrations of NO, this could represent an additional explanation for lower nNO in uncooperative younger children. Taken together, these results recommend prudential interpretation of low levels of nNO in children below the age of 6 months, in whom low nNO levels could represent a false positive finding. Moreover, low levels of nNO have been also reported in patients with cystic fibrosis,[27–32] sinusitis,[33–35] nasal polyps,[36,37] panbronchiolitis,[38] and HIV.[39] It is therefore clear that nNO measurement needs to be interpreted together with other relevant clinical findings (Table 1) and that this test will only exclude the diagnosis of PCD if nNO is elevated while low levels need to be carefully interpreted and used as an indication for selecting patients for nasal biopsy.[4] In order to better identify potentially PCD affected infants and to avoid inappropriate ultrastructural examinations, a follow-up of nNO levels could be helpful. In fact the data from the present study show that all the healthy children who had an initial low level when retested some months later showed significant increase in nNO values, whereas this was reported not to be the case for the subjects with PCD.[23]

Furthermore, there was no age effect in nNO levels in our patients with PCD, also confirmed by the repetition of the measurement after 6–8 months in the two non-cooperative children. Therefore, these results suggest that repeated measures are warranted when nNO is occasionally measured in young infants.

The limitation of this study was the sample size of patients with PCD, however, the ciliary dyskinesia is rare disease and further studies are required in order to evaluation nNO levels in uncooperative children. However, despite the small number of PCD subjects, the statistical analysis showed highly significant changes of the nasal NO levels in the different studied populations.

In conclusion, high levels of nNO are supportive of PCD diagnosis exclusion while low levels are indicative of the disease also in uncooperative infants when they remain persistently low and when other diseases have been excluded.

Conflict of interest

All authors (G.L. Piacentini, A. Bodini, D. Peroni, E. Rigotti, R. Pigozzi, U. Pradal and A.L. Boner) have not any potential, perceived, or real conflict of interest or financial arrangement (includes involvement with any organization with a direct financial, intellectual, or other interest in the subject of the manuscript).

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