Infant nasal nitric oxide over time: natural evolution and impact of respiratory tract infection

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Nasal NO is feasible in infants, increases from 0–2 years and is suppressed to PCD levels by respiratory infections http://ow.ly/7AsR30jPMSf

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ABSTRACT Nasal nitric oxide (NO) discriminates between patients with primary ciliary dyskinesia (PCD) and healthy individuals. We report feasibility of measurement and natural evolution of nasal NO and upon the impact of respiratory tract infection (RTI) on nasal NO in healthy infants (HI), followed from birth until age 2 years, with comparison to nasal NO in infant PCD.

Tidal-breathing nasal NO measurements were performed at scheduled visits at 2 weeks old and at 4, 8, 12, 18 and 24 months old, with extra visits during RTIs. Historical nasal NO measurements for infant PCD were included for comparison.

Altogether, 224 nasal NO measurements were performed in 44 enrolled infants. Median newborn nasal NO was 46 ppb (interquartile range (IQR) 29–69 ppb), increasing at a rate of 5.4% per month up to 283 ppb (IQR 203–389 ppb) at the age of 2 years. RTIs in 27 out of 44 infants temporarily suppressed nasal NO by 79%. Values for nasal NO in seven infants with PCD ranged from 6–80 ppb. The success rate to accept nasal NO sampling was 223 out of 224 measurements (99.6%).

Tidal-breathing nasal NO measurement was indeed feasible in infancy and nasal NO in HI increased significantly up to 2 years of age, in opposition to nasal NO in PCD cases, which stayed low past 2 years of age. RTI episodes caused marked, temporary reductions in nasal NO in HI indistinguishable from that in infant PCD, suggesting that nasal NO should be measured in RTI-free intervals.

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Introduction

Nitric oxide (NO) is produced throughout the airways, particularly in the paranasal sinuses [1]. It is a highly reactive molecule and has several roles in signalling within the lower airways; however, its function in the nose is not entirely clear. Nasal NO may be associated with host defense through a toxic effect on bacteria [2] and may influence ciliary beat frequency [3].

A considerable amount of nasal NO has been found in newborns despite their poorly developed and only partially pneumatized paranasal sinuses [4]. Furthermore, an age-dependent rise in nasal NO between infancy and school age, up to levels found in adults, has been demonstrated, probably reflecting the natural development and increasing pneumatization of the paranasal sinuses during this period [2].

Measurement of nasal NO in combination with high-speed video microscopy analysis and transmission electron microscopy (TEM) of respiratory epithelial samples is required for a diagnosis of primary ciliary dyskinesia (PCD) [5]. PCD is a genetic multi-organ disease, although it predominantly presents as a clinically heterogeneous group of respiratory ciliopathies, exhibiting reduced airway mucociliary clearance. Consequently, chronic respiratory disease manifestations and symptoms start from birth in more than 95% of patients [6, 7].

For unknown reasons, nasal NO is extremely low in patients with PCD when compared to healthy subjects and disease controls and low nasal NO is independent of the measurement modality [8]. Although a low level is only an indirect and suggestive indicator of PCD, nasal NO is considered useful as part of the diagnostic work-up for PCD in suspected cases [5, 9].

Although there is currently limited evidence-based treatment for patients with PCD [10, 11], an earlier age at diagnosis than the current mean of 5.3 years [12] is highly warranted because it may provide the potential to prevent and reduce early lung damage and other disease manifestations [13–16] that are involved in reducing quality of life in patients with PCD [17].

Measurement of nasal NO in infants and young children is possible using tidal-breathing nasal NO sampling [8, 18–20]. However, a high false-positive rate can be expected when mixed-age reference values are used [8]. A recent study provided some indications of the normal nasal NO level in infants under 12 months [21]. However, establishing specific age-corrected nasal NO reference values in a much larger population of healthy infants (HI) is still needed.

We hypothesised that nasal NO would increase substantially during infancy as previously indicated [2], drop markedly during respiratory tract infections (RTIs) and return to a predestined level at the next scheduled RTI-free measurement. Furthermore, we aimed to compare levels of nasal NO in HI and infants with PCD, hypothesising that HI nasal NO may quickly rise above PCD values and justify nasal NO as a potential discriminator between PCD and health even in infancy.

Consequently, this study had three aims. First, to study the natural evolution of nasal NO in HI by following them longitudinally with repeated nasal NO measurements from birth until 2 years of age; secondly, to study how ongoing RTIs affect the nasal NO levels during infancy; and thirdly, to compare nasal NO between HI with and without ongoing RTIs and infants with PCD to further investigate its role in early diagnosis of PCD. Some of the results of this study have been previously reported in the form of an abstract [22].

Materials and methods

Design

This was an investigator initiated, prospective, observational, longitudinal, single-centre study, with no direct or indirect sponsorship from manufacturers of equipment. Six visits were intentionally scheduled for each participating infant: at age <2 weeks and again at 4, 8, 12, 18 and 24 months of age, given an absence of any RTI the past 14 days. Parents were allowed to reschedule planned visits if necessary in an attempt to diminish the drop-out rate. All were invited for optional extra nasal NO measurements during episodes of RTI during the 24-month study period. Historical tidal-breathing nasal NO data from infants with PCD was additionally included for comparison.

Subjects

Full-term HI without perinatal or neonatal complications were recruited from the maternity ward. Standard birth and neonatal data were collected at inclusion. Patients with a positive diagnosis of PCD in infancy and first tidal-breathing nasal NO measured before 2 years of age were additionally included.

Measurements

For measurements of nasal NO, we used a stationary chemiluminescence analyzer (CLD 88sp NO-analyzer; ECO MEDICS AG, Duernten, Switzerland) with online measurement and real-time screen displays of results.

Tidal-breathing nasal NO sampling was performed while the child was placed on the lap of a parent or sleeping on their back. A nasal probe with a central lumen connected *via* a tube to the NO-analyzer was gently inserted into one nostril. Tidal-breathing nasal NO sampling was performed during relaxed tidal breathing *via* low continuous suction at a sampling flow rate of 0.33 $L \cdot min^{-1}$ using the averaged nasal NO value from the three highest, distinct, visible peak concentrations measured within 30 to 40 s and read directly as point values on the screen.

Measurements were performed in all subjects on several occasions by one experienced observer. All participants were expected to have triplet data sets for nasal NO at each visit. In case of known anatomical defects, nasal bleeding, or previous nasal surgery the affected side was avoided and the opposite nostril chosen for measurements. No concurrent local nasal medication was allowed. Ambient NO was registered before all measurements and tidal-breathing nasal NO measurement was never performed while the infant was crying.

Ethics

Prior to participation, the parents gave written informed consent. The study was approved by the local ethics committee of the Capital Region of Denmark (journal no. H-C-2008-036).

Statistics

To assess the natural evolution of nasal NO in HI over time and how RTIs affected nasal NO levels, linear mixed models were applied [23]. The nasal NO measurements were log transformed to meet the assumption of normality and homoscedasticity. For infants older than 2 weeks, a linear relationship between age and log nasal NO measurement was found. Measurements taken during RTI exhibited lower levels but the same linear relationship corresponding to a regression model with two parallel lines. The routine measurements taken before 2 weeks did not follow the same line as the routine measurements taken after 2 weeks of age. This was handled by extending the linear regression model with a separate intercept term for measurements before 2 weeks, corresponding to a model where the mean of measurements taken before 2 weeks is constant and the mean of measurements taken after 2 weeks increases linearly with age (with different intercepts depending on RTI). We further investigated whether routine measurements following an RTI measurement were suppressed by including an extra variable indicating whether an RTI measurement had been taken up to 2 months before. The random part of the model included a random intercept for each child. Analyses were performed using SPSS version 23, R version 3.2.0 and SAS version 9.4. Any p-values less than 0.05 were considered significant.

Results

We included 44 newborn HI up to 2 weeks of age who were scheduled for longitudinal tidal-breathing nasal NO measurements at six visits within 0–2 years of age, including extra measurements during RTIs.

Tidal-breathing nasal NO during RTI was measured in 27 out of the 44 infants. RTI measurements were made repeatedly in the same child if repeated episodes of RTI occurred during the 2 years of observation. Thus, an additional 61 measurements were performed during RTIs; however, no RTI measurements were performed during the first 2 weeks from birth. Historical tidal-breathing nasal NO data from seven patients with PCD were included for comparison using the first measurement performed at less than 2 years of age and follow-up nasal NO data where available.

Ambient NO was rarely above 5 ppb, and never above 10 ppb in the study. None of the HI had respiratory distress at birth requiring perinatal resuscitation or continuous positive airway pressure (CPAP) treatment and none had been transferred to the neonatal intensive care unit (NICU). None of the included children showed any clinical features of neonatal distress. Specifically, none had signs indicative of PCD or cystic fibrosis (CF) during the study period, although neonatal screening for CF was not implemented in Denmark at the time of the study. Descriptive characteristics are given in table 1.

From our Danish National PCD cohort consisting of 130 patients, we retrieved historical data for seven infants (5% of the cohort) that had nasal NO measured before the age of 2 years. All had classic PCD symptoms: impaired ciliary beat function and ciliary ultrastructural defects shown by TEM and immunofluorescence analysis. Furthermore, three patients were genetically characterised with either a CCDC151 loss-of function mutation (n=1) or a CCDC39 loss-of-function mutation (n=2). Diagnostic characteristics are given in table 2.

For HI older than 2 weeks, a linear association between age and log-transformed nasal NO was found for scheduled and RTI measurements. The linear association did not differ for scheduled and RTI measurements ($p=0.44$ by test of equal slopes) but the RTI measurements demonstrated lower nasal NO levels ($p<0.0001$) for all ages. Measurements taken at less than 2 weeks old, all scheduled, demonstrated

TABLE 1 Characteristics of the healthy infants included in the study

Characteristics	Results
Male sex	23 (52.3)
Gestational age weeks	40.3 (36.6–43.6)
Birth weight g	3600 (2410–4400)
Birth length cm	52 (47–59)
Age at first nasal NO measurement days	5 (1–14)

Data are presented as n (%) or median (range). Total number of subjects is 44. NO: nitric oxide.

lower levels than suggested by the linear association between age and scheduled nasal NO measurements for ages above 2 weeks ($p<0.0001$). An age trend was not found during the first 2 weeks from birth ($p=0.10$). The estimated parameters are given in table 3 and the model is illustrated in figure 1.

In HI >2 weeks old, nasal NO increased by 5.4% per month (95% CI 3.7–7.0%; $p<0.0001$) (table 3 and figure 1). RTIs in 27 out of 44 HI temporarily suppressed nasal NO by 78.9% (95% CI 73.4–83.2%). Although nasal NO during an RTI was markedly suppressed, we did not find decreased nasal NO for measurements taken within 2 months of a RTI measurement ($p=0.88$). An example of a profile plot for one child is shown in figure 2, illustrating the longitudinal trend for scheduled measurements and the drop in nasal NO during a RTI. Profile plots for each of the 44 included children are given in the supplementary material (figure E1).

Historical nasal NO values in seven infants with PCD were low within the first 2 years of life (6–80 ppb), yet similar to nasal NO in HI during a RTI, as shown in figure 1. Moreover, we found overlapping nasal NO values for HI without clinical signs of RTI and infants with PCD up to the age of 12 months. Follow-up measurements later in childhood showed that nasal NO values remained low in the children with PCD (table 2).

A cut-off tidal-breathing nasal NO value of 158 ppb was previously established in a mixed group of children and adults [8]. In the current study 15 out of 16 HI (94%) had tidal-breathing nasal NO above this value at 2 years of age. The success rate for acceptance of measurement among the HI was high (223 out of 224; 99.6%). The nasal NO measurements were easily performed during sleep after breast-feeding or bottle-feeding in newborns less than 2 weeks old and in HI who were 3–4 months old. HI older than 4 months were the most reluctant to comply and usually required repeated measurements for success, without having the child pull out the nasal probe before sampling was finished. Despite such issues, nasal NO measurements were performed successfully in all HI except for one 8-month old infant who completely refused the nasal probe. HI older than 1.5 years could usually be persuaded verbally to accept the nasal probe (with or without distraction by their parent) long enough for a complete sampling.

TABLE 2 Diagnostic characteristics of seven infants with a positive diagnosis of primary ciliary dyskinesia (PCD) and their nasal nitric oxide (NO) concentrations measured during infancy and at follow-up

PCD patient	Sex	Ciliary function	Ciliary ultrastructural defects	Immunofluorescence	PCD mutations[#]	First measurement		Follow-up measurement	
						Nasal NO ppb	Age months	Nasal NO ppb	Age months
1	Male	Immotile	ODA defect	Unknown	Unknown	35	1	23	7
2	Female	Stiff, slow and asynchronous	Central pair microtubule defect	Unknown	Unknown	40	4	30	4.5
3	Male	Immotile	ODA defect	ODA (*DNAH5* negative)	Unknown	15	3.5	4	71
4	Male	Slow and asynchronous	Tubular disorganisation with IDA	N-DRC + IDA (*CCDC39*, *LRRC48* and *DNALI1* negative)	CCDC39:c.610-2 A>G heterozygote	6	17		
5	Male	Slow and asynchronous	Tubular disorganisation with IDA	N-DRC + IDA (*DNALI1* negative)	CCDC39:(p.E851X) + splicing mutation	80	12.5	80	145
6	Female	Immotile	ODA defect	ODA (*DNAH5*, *ARMC4*, *CCDC114* and *CCDC151* negative)	CCDC151:c.925 G>T p.Glu308	11	2.5	30	134
7	Male	Immotile	ODA defect	ODA (*DNAH5* negative)	Unknown	8	0.5	108	25

Nasal NO was very low in newborns <2 weeks old (median ppb value: 46 (IQR 29–69)) but increased markedly with age (median ppb value: 283 (IQR 203–389) at an age of 2 years). See table 4 for further information. [#]: all patients were repeatedly investigated for mutations. ODA: outer dynein arm; IDA: inner dynein arm; N-DRC: nexin–dynein regulatory complex; IQR: interquartile range.

TABLE 3 Statistical model estimates

Parameters	Linear mixed model			Interpreted estimates	
	Estimate[#]	95% CI	p-value	Estimate[¶]	95% CI
Mean parameters					
Below 2 weeks of age					
Newborn scheduled measurement (intercept)	3.679	3.457–3.900	<0.0001	39.6 ppb	31.7–49.4 ppb
Above 2 weeks of age					
Infant scheduled measurement (intercept)	4.276	4.052–4.500	<0.0001	71.9 ppb	57.5–90.0 ppb
Infant RTI measurement	−1.556	−1.786 to −1.326	<0.0001	−78.9%	−83.2 to −73.4%
Infant age months	0.052	0.037–0.068	<0.0001	5.4%	3.7–7.0%
Variance parameters					
Between-subject variance (random intercept)	0.068	0.029–0.308	0.04		
Within-subject variance (residual error)	0.49	0.400–0.607	<0.0001		

RTI: respiratory tract infection. [#]: estimated parameters from the linear mixed model used for analysis of log-transformed nasal nitric oxide (NO). Newborn babies only had scheduled measurements. For healthy infants (HI) >2 weeks old only, the model included RTI (yes/no) and age (months). A random intercept for each child was used in the random part of the model. [¶]: estimates transformed to the nasal NO scale (a ratio scale, determined as the antilog of the estimated mean parameters, exp(estimate)). Intercepts are to be interpreted as median values whereas differences between groups are reported as percentages.

Out of a total of 264 scheduled visits, parents met up for a total of 163 scheduled nasal NO measurements, thus resulting in an overall adherence rate of 62%. The adherence to meet for scheduled visits decreased with the age of the children. By 12 months of age, 23 (52%) had dropped out and by the end of the study only 16 (36%) completed the 24-month measurement. In addition, adherence to scheduled visits at the intended age became less strict over the study period and the actual measurements became more dispersed, especially around the intended 12-month and 18-month measurements (figure 1). Adherence data are given in table 4.

Discussion

We present the first longitudinal study describing the natural development of nasal NO in HI and demonstrate that nasal NO increases significantly at a fixed rate of 5.4% per month up to 2 years of age. Infants with PCD did not follow this increasing trend but had nasal NO levels which stayed low past infancy. We also show that RTIs resulted in a marked but temporary drop in nasal NO and that these suppressed values were similar to nasal NO levels in infants with PCD of the same age. Despite choosing a challenging age group for nasal NO measurements, our success rate of 99.6% shows that it is indeed feasible to measure nasal NO successfully throughout the first 2 years of life.

Nasal NO is extremely low in patients with PCD and nasal NO measurement has been part of the PCD work-up algorithm for the past 10 years in some European centres, including the UK [24] and Denmark,

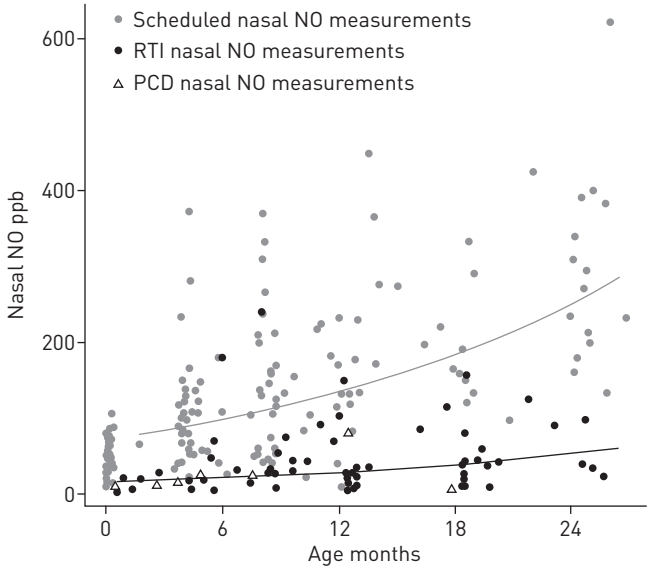

FIGURE 1 Natural evolution of nasal nitric oxide (NO) levels from birth to 2 years of age in 44 healthy infants (HI). The lines illustrate the estimated median nasal NO values for scheduled measurements (grey line) and for measurements during respiratory tract infections (RTIs) (black line) for infants older than 2 weeks. Grey dots represent scheduled nasal NO measurements in HI, while black dots show nasal NO measured in the same HI during RTIs. Triangles show nasal NO in infants with primary ciliary dyskinesia (PCD).

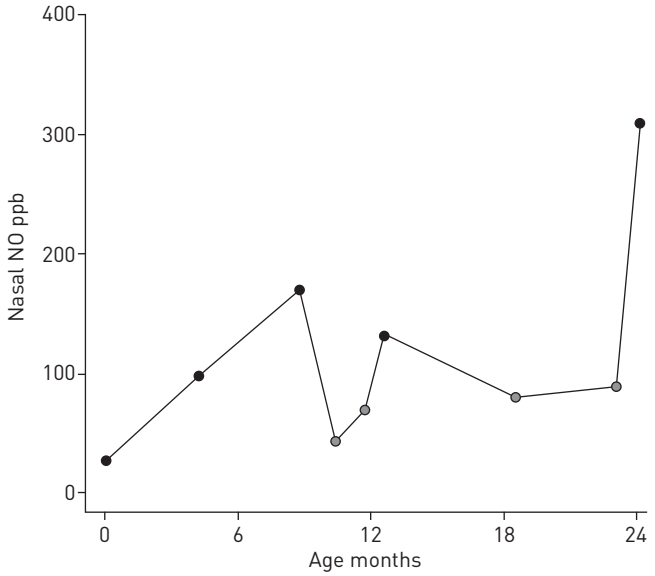

FIGURE 2 Example of a longitudinal series of nasal nitric oxide (NO) measurements from birth to 2 years of age in one healthy infant (HI), showing increasing nasal NO with age for scheduled measurements (black dots) but temporarily reduced nasal NO levels during respiratory tract infection (RTI) episodes (grey dots).

and has been included as a PCD screening tool in the European PCD guidelines of 2009 [25]. The American Thoracic Society (ATS)/European Respiratory Society (ERS) recommend the velum closure technique [26] for nasal NO and the North American Genetic Disorders of Mucociliary Clearance Consortium recommends criteria for an acceptable nasal NO sampling manoeuver that include a greater than 20 s exhalation and a steady NO signal plateau for 3–10 s during sampling [27]. However, meeting such standardised criteria is not feasible in young children. Piacentini *et al.* [18] previously found that 250 out of 300 healthy children between 1.5 and 7.2 years of age were unable to perform velum closure sampling as recommended by the guidelines. Leigh *et al.* [27] concluded that velum closure nasal NO measurement cannot be performed in children younger than 5 years of age. Hence, the current standardizations requiring velum closure need to be challenged if nasal NO measurements for PCD work-up are to include infants and young children. Currently, no guidelines exist for guiding the sampling of tidal-breathing nasal NO; however, tidal-breathing nasal NO is the most obvious choice for nasal NO sampling in infants and young children due to its feasibility and since it discriminates equally for PCD despite tidal-breathing nasal NO values generally being lower than in velum closure nasal NO [8, 19, 28].

Adams *et al.* [21] recently published normative values for nasal NO from a cross-sectional study in 42 HI less than 1 year of age and compared these with nasal NO in 15 infants with PCD. In concordance with our results, they showed very low median nasal NO values of approximately 40 ppb in HI less than 2 weeks old and a marked increase up to 12 months of age reaching approximately 200 ppb. Similar to our study, the majority of infants with PCD had abnormally low nasal NO, thereby supporting our suggestion that nasal NO measurements could be used to guide PCD assessments in infants.

The mechanism explaining occasional low nasal NO in subjects without PCD also remains unclear, but is often reported to coexist with paranasal sinus infections. Hence, trapped NO in obstructed paranasal sinuses or in mucus biofilms may partly explain the low nasal NO in patients with CF and in patients without CF

TABLE 4 Longitudinal tidal-breathing nasal nitric oxide (NO) measurements scheduled at <2 weeks and at 4, 8, 12, 18 and 24 months of age in 44 included healthy infants

Subjects n	44	39	31	21	12	16
Intended age at measurement	<8 days	4 months	8 months	12 months	18 months	24 months
Actual age at measurement	1–14 days	3.6–6.3 months	7.6–9.8 months	10.4–14.2 months	15.2–21.0 months	22.2–27.1 months
Tidal-breathing nasal NO$^{\#}$ ppb	46 (29–69)	98 (56–130)	129 (60–200)	171 (110–228)	178 (137–261)	283 (203–389)
Tidal-breathing nasal NO$^{\#}$ nL·min^{-1}	15 (9.6–22.8)	32 (18.5–42.9)	42.6 (19.8–66)	56.4 (36.3–75.2)	58.7 (45.2–86.1)	93.4 (67.0–128.4)
Adherence to scheduled measurements %	100	88.6	70.5	47.7	27.3	36.4

Data is presented as median (IQR) unless otherwise stated. $^{\#}$: sampling flow rate was 0.33 L·min^{-1} (conversion: nasal NO (ppb)×flowrate (L·min^{-1})=nasal NO (nL·min^{-1})).

and PCD who have chronic sinusitis [29] or paranasal polyposis [30]. In addition, low nasal NO has been reported in a small number of patients with diffuse panbronchiolitis [31], a disease in which inflammation occurs in the respiratory bronchioles in combination with bronchiectasis and recurrent sinusitis.

To date, no longitudinal studies have evaluated nasal NO levels during ongoing, simple viral and/or bacterial RTIs in young otherwise healthy children. As such, our results for these measurements cannot be compared with any previously published data. We speculate that the very marked drop in nasal NO during an RTI to levels seen in infants with PCD is related to an acute nasal blockage by mucus and nasal inflammation, thereby temporarily mimicking the persistent nasal/paranasal conditions existing in PCD.

During RTI episodes, the 78.9% decline in nasal NO values appeared as a parallel-shifted trend at a lower level compared to natural evolution but still increasing at the same rate of 5.4% per month as seen for scheduled nasal NO results in RTI-free periods. Between RTI episodes, nasal NO values were markedly higher in HI compared to patients with PCD at all time points. As such, our study suggests that nasal NO may discriminate between HI and PCD cases and emphasises that nasal NO measurements for PCD diagnostics should always be performed during RTI-free intervals.

Since we also found some examples of low levels of nasal NO in HI in RTI-free intervals that overlapped with PCD, our results underline that nasal NO cannot stand alone as a single test. We recommend that nasal NO measurements in infants are repeated after 2–3 months even in the absence of clear signs of infection and that further diagnostic testing should always be applied for full PCD work-up.

In this study, at 2 years of age the majority of HI had reached tidal-breathing nasal NO concentrations above the cut-off value previously established to discriminate between PCD and health (158 ppb, 52.1 $nL \cdot min^{-1}$) [8]. We suggest that distinct tidal-breathing nasal NO reference values should be generated at least up to the age of 2 years. Mixed-age cut-off values may be applied after approximately 2 years of age if using a tidal-breathing sampling technique.

Limitations of the study

The study was a single centre study with a limited sample size. Tidal-breathing nasal NO is the necessary choice of sampling modality if infants are to be included for nasal NO measurements and has been shown to be highly feasible in this infant population, with a success rate of 99.6%. However, patience during sampling was crucial and sometimes several tries were needed. In addition, a high false-positive rate (39%) has previously been described for children below 6 years of age when using a cut-off value of 158 ppb in tidal-breathing nasal NO measurement to separate PCD patients from healthy patients in a mixed-age cohort [8].

Establishing cut-off nasal NO values to discriminate between infants with or without PCD is certainly warranted, but is also a major challenge since nasal NO increases rapidly in this age group. This requires large numbers of infants with PCD to assure sufficient power; however, the number of known infants with PCD is very limited because most PCD patients are diagnosed later in childhood, as shown in a previous European survey including 1009 patients with PCD across 26 European countries (223 centres) where the median age at diagnosis was 5.3 years [12]. In our cohort, we only found seven out of 130 patients (5%) with PCD who had nasal NO measurement at age less than 2 years.

Adherence to attendance at scheduled visits was poor after 12 months from inclusion, as more than half the children were lost to follow-up at this point. The large dropout rate after 12 months can partly be explained by the fact that many Danish mothers return to their jobs when their child reaches 12 months of age and the families have less flexibility thereafter. Less family flexibility after 12 months of age may also explain why adherence to the intended appointments became less strict. As this kind of absence is not related to the nasal NO level but rather to age, absence can reasonably be assumed to be "missing at random", in which case linear mixed models provide a valid tool for inference [32].

Due to the design of the study, extra RTI-event measurements were only performed if the parents actively reported the need. As such, there must inevitably be missing RTI events in the study and, in a future study, a more controlled execution could probably increase adherence and thus provide stronger data. However, despite this limited data, the suppression of nasal NO during RTI was shown to be consistent and highly significant.

In conclusion, nasal NO can be measured in all age groups (including newborn babies) using tidal-breathing nasal NO sampling, with a very high rate of success in accepting measurement. The very low nasal NO values at the beginning of life increased naturally with age and hence the ability to discriminate PCD from health also increased. However, during RTI episodes nasal NO levels were markedly suppressed (to the same levels as seen in infants with PCD). We recommend that separate nasal NO reference values be generated up to at least 2 years of age and that nasal NO for PCD work-up always is measured during RTI-free intervals.

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Conflict of interest: None declared.

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