CHOICE OF NASAL NITRIC OXIDE TECHNIQUE AS FIRST LINE TEST FOR PRIMARY CILIARY DYSKINESIA

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ABSTRACT (word count: 200 words)

Background: Nasal nitric oxide (nNO) has a well-known potential as indirect discriminative marker between patients with primary ciliary dyskinesia (PCD) and healthy subjects, but real-life experience and usefulness in young children is sparsely reported. Three nNO sampling methods were examined and compared as first-line tests for PCD.

Materials and methods: Healthy subjects, confirmed PCDs, consecutive referrals with PCD-like symptoms and patients with cystic fibrosis (CF) had nNO sampled during breath hold (BH-nNO), oral exhalation against resistance (OE-R-nNO), and tidal breathing (TB-nNO) aiming to expand age range into infancy.

Results: 282 subjects, 117 consecutive referrals, 59 PCDs, 49 CF patients and 57 healthy were included. All methods separated significantly between PCD and non-PCD including CF with reliability in ranking order BH-nNO > OE-R-nNO > TB-nNO. Acceptability in children ranked in reverse order. A problematic high fraction (39%) of false positive TB-nNO was found in young children. An unexpected large fraction (6.8%) of PCDs had nNO values above cut-off.

Conclusion: Nasal NO is a helpful first-line tool in real-life PCD work-up in all age groups if sampling method is chosen according to age. nNO can be misleading in a few patients with true PCD. Further studies are strongly needed in young children.

Key words: Children, methods, nasal nitric oxide, Primary ciliary dyskinesia
INTRODUCTION

Primary ciliary dyskinesia (PCD) is an inherited disorder characterized by abnormalities in ciliary structure and/or function, impaired mucociliary clearance, and recurrent or chronic airway infections[1] which are a serious threat to lung function, probably even in young children[2]. Early diagnosis is important, since lung function may improve or stabilize after diagnosis and initiation of treatment[2], and may be achieved by support from measurement of nasal Nitric Oxide (nNO)[3;4]. nNO is non invasive, painless, easily performed from school age, and has the capacity to discriminate between patients with PCD and healthy subjects in highly selected well-characterized populations[3-6]. So far, nNO has been reported to be very low almost exclusively among subjects with PCD, and to separate convincingly between the presence and absence of PCD[3-8]. However, overlap in nNO values have been reported between PCD and other pulmonary diseases with symptoms mimicking PCD[9-11]. Hence, the true discriminative power of nNO in less selected, mixed population of patients referred for diagnostic testing of PCD will need to be settled before the role of nNO in the diagnosis of PCD can be fully appreciated.

Different techniques have been proposed, but no one technique fits all age groups. Recommendations for measurements have been provided[12], stressing the need for soft palate closure during sampling so as to avoid dilution from lower airways, but the standardization of methodology is still lacking. Different manoeuvres during sampling have been evaluated[13-15], but most techniques are not applicable to infants and young children.

We hypothesized that nNO measurements, if adjusting sampling method according to age, could separate between patients with and without PCD in a real-life setting of consecutively referred patients in all age groups, however with the expectation of a less convincing separation.
The aim of this study was to evaluate 3 different sampling methods for nNO-measurements as first line test in a real-life setting of patients consecutively referred for PCD work up, with the intention of expanding the age range for nNO measurements to include young children and infants with a view to achieve early PCD diagnosis.
MATERIALS & METHODS

Study subjects

Four groups of subjects, children and adults, were included:

1. Patients with confirmed PCD from the National Danish PCD cohort [2] with consistent history and ongoing symptoms of PCD, and abnormal ciliary beat pattern and/or frequency (CBP and CBF). Additionally, Electron Microscopy (EM) and measurements of Pulmonary Radioaerosol Mucociliary Clearance (PRMC)[16] had been performed in 78% and 68% of the patients.

2. Patients referred for PCD work-up. Patients with clinical symptoms and history suggestive of PCD, consecutively referred from secondary paediatric and adult centres. Exclusion of CF and immunodeficiency was part of the initial work up.

3. CF patients documented by sweat and genotype testing were recruited from the cohort of CF Centre Copenhagen.

4. Healthy non-smoking Subjects (HS) were recruited among staff members and the children of staff.

All subjects gave their informed consent prior to participating in the study. The study was approved by the local ethics committee (KF 01-045/04).

Study design

The study consisted of 3 sub-studies:
Sub-study 1 aimed to evaluate three different sampling methods for nNO-measurements with the intention of expanding the age range for nNO measurements to include young children and infants. It was a cross-sectional study of children and adults with confirmed PCD or CF and healthy controls. Within-occasion repeatability, long-term repeatability, acceptability, agreement between methods, dependency of age, and subject category on repeatability and normative levels were assessed.

Sub-study 2 aimed to investigate the discriminative power of each of the three methods evaluated in sub-study 1, determining sensitivity and specificity of cut-off values calculated to separate between presence and absence of PCD. It was a cross-sectional study of HS and selected patients with confirmed PCD.

Sub-study 3 aimed to test the three nNO sampling methods in a real-life setting in order to test their power to separate between PCD and non-PCD patients in a mixed cohort of patients with PCD-like symptoms consecutively referred for PCD work up. It was a prospective study applying cut-off values from sub-study 2. Final diagnostic work-up in terms of CBP, CBF and EM was performed \textit{post hoc} to nNO measurement.

\textbf{Methods}

\textbf{Nasal NO measurement:}

Online nNO measurements were performed using NIOX® (Nitric Oxide Monitoring System, Aerocrine, Sweden) equipment. Nasal NO gas was aspirated via a nasal olive probe inserted to one nostril by use of passive sampling flow rate of 5 mL/s (~ 0.3 L/min), according to recommendations[12]. Prior to nNO measurement the subjects were interviewed about anatomical
defects of the nose and sinuses. If the subject reported of known nasal defects from one side, the normal side was chosen.

Three measurements were obtained at each session and the mean value was used as result. Outlying values, not further defined, were discarded at the discretion of the technician.

Measurements were postponed in case of acute upper and lower airway infections within 2 weeks prior to nNO measurement.

Any concurrent use of nasal decongestives or nasal steroids were unchanged during follow up in sub-study 1.

*Three sampling methods were applied:*

1. Breath Hold (BH-nNO) sampling. This method assured soft palate closure and stable plateau values for at least 10 seconds, in alignment with ATS/ERS recommendations[12].
2. Oral exhalation against resistance (OE-R-nNO). By use of a “party-blow-out-toy”, temporary soft palate closure was accomplished during the active blowing[17]. The subjects were instructed to blow out in order to unfold the “party-blow-out toy” and keep it inflated for as long as possible, thereby creating plateaus during 4 to 5 seconds of maximal nNO concentration.
3. Tidal breathing (TB-nNO): normal relaxed tidal breathing was allowed during sampling. No soft palate closure is achieved by this method. Mean values were calculated from the highest three distinct visible peak concentrations, read directly as point values on the screen.
For each subject the choice of sampling method was adjusted according to age and level of cooperation. BH-nNO was not attempted in children below 4 years of age. If cooperation with BH-nNO was anticipated in a given child, this was the first method of choice. If cooperation or technique then failed (i.e. breath hold was not possible for the child or no stable plateaus were seen), the BH-nNO results were excluded and the next method in line, OE-R-nNO, was attempted. Again, if cooperation or technique failed (inability to blow the “party-blow-out-toy” sufficiently, or lack of plateaus) the results were omitted and the last method, TB-nNO, was performed. Thus, a formal acceptability study was not performed.

**Confirmative PCD diagnosis**

Diagnosis of PCD depended upon combined analysis of CBP- and CBF-, and ultrastructure analysis as per ERS Task Force consensus[1]. CBP and CBF were analysed in 5 to 10 nasal epithelial strips in a patient, using an interference contrast microscope (Leica DMLB; Leitz; Stuttgart, Germany) and digital frame-by-frame assessment of ciliary movements. EM pictures of at least 100 ciliary cross sections were evaluated for ultrastructural defects. Counts of outer and inner dynein arms were performed in a fraction of at least 10 cross sections.

**Analysis**

Median, range, mean, and standard deviations were applied for baseline characteristics. Reliability of each sampling method was evaluated as CV% for all subjects. Repetitability between occasions was evaluated according to Bland and Altman[18] in a random sample of patients. Box-and-Whisker-plots were used to demonstrate the separation between subject groups for each sampling method. In comparison of methods, limits of agreements (LoA) of OE-R-nNO and TB-nNO were
determined against BH-nNO as the reference method. An unpaired t-test was used for comparison of mean nNO-values between groups. Wilcoxon Rank Sum Test was applied when numbers were small. A p-value < 0.05 defined the level of statistical significance.

RESULTS

In total, 282 subjects were included. Characteristics and distribution between subject groups are given in Table 1. One hundred and seventeen patients were consecutively referred for PCD work-up of which 20 (17.1%) were diagnosed with PCD. Ambient NO during measurements in 106 subjects was 19.9 (0.1 to 63.6) ppb.

Sub-study 1
Normative data, mean (SE), ranged from BH-nNO: 908 (33) ppb to OE-R-nNO: 788 (41) ppb to TB-nNO: 534 (30) ppb (Figure 1, Figure 2, Figure 3). Overall repeatability, CV% (SD), within occasion was 6.7% (8.8), 10.4% (14.2) and 12.3% (15.0) for BH-nNO, OE-R-nNO and TB-nNO, respectively. Among children below 6 years of age, CV% of TB-nNO was 10.8% (9.8) and thus age independent. Within occasion repeatability of BH-nNO differed significantly between PCDs (9.7%) and HS (3.6%) (p<0.0001), whereas no difference was shown between non-PCD referrals (6.3%) and HS (3.6%) or between referrals with PCD (10.6%) and confirmed PCD patients (9.7%). CV% in CF was 4.8%.
OE-R-nNO was in closer agreement with BH-nNO than TB-nNO. The 95% LoA between methods irrespective of diagnosis were 190.2 to -166.9 ppb, mean (SD) difference 11.7 ppb (178.5) (BH-nNO vs. OE-R-nNO) and 572.2 to –182.9, mean (SD) difference 194.7 (377.5) (BH-nNO vs. TB-nNO).
Long-term repeatability assessed as mean nNO differences and LoA within variable time intervals (Online Depository Table A) showed comparable 95%CI limits of all three methods.

Acceptability in children was judged from the proportion of subjects less than 6 years of age (n=62) having performed each test successfully and the age range of such subjects. As such, the acceptability of TB-nNO was 95.2% with a minimum age of 14 days, thus highly exceeding acceptability of OE-R-nNO (25.8%, minimum age: 2.5 yrs) and BH-nNO (3.2%, minimum age: 4.1 yrs).

Sub-study 2

All methods discriminated significantly between PCD and HS (Figure 1, Figure 2 & Figure 3, and Table 2). However, unexpectedly high nNO values were seen in 4/59 (6.8%) of confirmed PCD patients, resulting in overlap between PCD and non-PCD.

Cut-off values with sensitivity/specificity/Area Under ROC Curve (AUC) providing the best discrimination between PCDs and HS were 175 ppb/91.1/100/0.95 for BH-nNO, 242 ppb/94.3/100/0.97 for OE-R-nNO, 158 ppb/94.4/100/0.97 for TB-nNO, and are further specified in Online Depository Table B and Online Depository Figure A, Figure B & Figure C.

nNO provided significant discrimination between PCD and CF patients in all methods (Figure 1, Figure 2 & Figure 3, and Table 2). However, despite significant discrimination, overlap independent of age occurred (Figure 1, Figure 2, Figure 3 and Online Depository Figure D, Figure E & Figure F).

Sub-study 3
Cut-off values from sub-study 2 were applied in the mixed cohort of 117 referrals (Online Depository Table C), and the significant discrimination between PCD and non-PCD was completely mirrored in this unselected group of referrals. A solid diagnostic capacity was demonstrated by both BH-nNO and OE-R-nNO, and confirmed by high values of both sensitivity and specificity, whereas TB-nNO demonstrated poor specificity (0.8) due to a high number (n=17) of false positives (Online Depository Table D).

Referrals without PCD and false positive TB-nNO were all children, median age (range) 2.7 (0.2 to 6.3) years. The proportion of false positives was 39% in children below 6 years, thereby correctly excluding 61% of referrals without PCD in this age group. None of these patients used nasal decongestives or nasal steroids.

During the study period we diagnosed PCD in 2 referred infants: a 16 day-old boy with TB-nNO of 9 ppb and a 8 week-old girl with TB-nNO of 11 ppb.

One in twenty referrals with later confirmed PCD had above-cut-off nNO.

The overall number of false negatives (n=5) among the PCD patients was substantial compared to previous studies. Clinical and diagnostic features of these patients with nNO values above cut-off are summarized in Table 3. None of these patients were atopic. Their FeNO was normal (range 8 to 18 ppb).
DISCUSSION

This is the first large prospective study evaluating the usefulness of nNO measurement as a first line test for PCD in an unselected cohort of PCD-referrals with age range between infancy and adulthood. Our study confirmed earlier retrospective reports[3;4;7] in favour of nNO having a frontline role in the work-up for PCD. We examined 3 different sampling methods (BH-nNO, OER-nNO and TB-nNO), each intended for different age groups and levels of cooperation. All techniques demonstrated acceptable reliability, agreement, and diagnostic capacity in addition to comparable long-term repeatability. This proves promising for the expansion of the age range of nNO measurements to include young children and infants. nNO levels in infant PCDs are so far largely uninvestigated and only published in a few case reports as extremely low[19-21]. We add to this literature two infants with TB-nNO values of 9 ppb and 11 ppb, respectively.

Importantly, we report for the first time a remarkable number (n=5) of PCD patients with high nNO levels (540 to 1486 ppb) and an incidence of above cut-off nNO levels reaching 6.8%. High or normal nNO values in PCD is a phenomenon otherwise only very rarely reported[4;5]. Diagnosis of PCD may be difficult because secondary functional and structural abnormalities due to actual or recent infectious disease can be misinterpreted as primary disease[1]. In our study, all PCD patients demonstrating high nNO were re-evaluated for their clinical features and ciliary functional abnormalities. Furthermore, EM-tests were repeated in 3 out of 5 cases and demonstrated identical ultrastructural abnormalities in the second test. All in all these patients were confirmed to be “true PCD patients,” despite normal nNO. Clinical characteristics, as well as ciliary ultrastructural defects, varied greatly and did not give the impression of any common features. Notably, only 1 in 5 had situs inversus and none had chronic sinusitis. Whether absence of chronic sinusitis could be
part of the explanation of high nNO in PCD remains to be illuminated, and further studies will be needed.

The attempt to evaluate nNO measurement in a real-life setting clarified some limitations regarding measurements in young children. TB-nNO could be measured without lower age-limit, but led to progressive loss in power in terms of within-subject reliability and specificity. Thus we report a considerable fraction of false positive cases among young children measured by TB-nNO. Although CV% was comparable in TB-nNO measured in children <6 years (10.8%) and all age groups (12.3%), indicating that young children could repeat TB-nNO values as good as any individual, the overall CV% of TB-nNO was relatively high and agreement poor compared to BH-nNO.

TB-nNO is weakened by the lack of soft palate closure during sampling, where peak-values are read directly as single points on the screen, as opposed to better-defined plateaus provided by BH-nNO and OE-R-nNO sampling.

The high rate of false positive young children definitely represents a major weakness of TB-nNO. However, TB-nNO is a fast, easy, painless, non-invasive, and well-accepted test in young children, and therefore has clear advantages compared to other tests involving either radiation[22] or high demands of cooperation[23]. TB-nNO also demonstrated a comparable negative predictive value (0.99) compared to BH-nNO (0.98), and is thereby equally strong in ruling out PCD in cases with above-cut-off values, e.g. we were able to exclude nearly two-thirds of our non-PCD referrals below 6 years of age by TB-nNO cut-off. TB-nNO is therefore suggested as a supplementary method in referrals not able to cooperate to either BH-nNO or OE-R-nNO. The expectation from TB-nNO should be to exclude a fraction of non-PCD referrals with above-cut off values from further investigation, with a view to minimize the amount of patients in need of further
conventional ciliary function and ultrastructural analyses, which are both painful and overall time- and resource-consuming.

Normative nNO data in infants and young children are largely lacking as only very few studies exist, and contain very small groups of healthy children[5;17;24]. Specific reference values for this age group are important as non-PCD infants have also been shown to exhibit very low levels of nNO, most probably caused by their undeveloped paranasal sinuses[19;25;26].

In this study, we present normative data in HS above 3 years of age thereby probably setting cut-off values too high when applying these reference values to young children. Further studies are clearly needed to establish cut-off values for TB-nNO measurements in infants.

Previous studies in healthy subjects reported CV% of repeated measurements to be dependent on age [24] and specific sampling technique[27]. The CV% of BH-nNO in a population of HS of all age groups was 3.6% and comparable to levels found in previous studies [24;27]. However, in PCDs we found significantly higher CV% probably explained by the low absolute values in PCD, which is in alignment with Karadag et al.[4].

Acceptability of nNO measurements in children has only been addressed in few studies[20;28]. In one study, technical failure was found in 15% of 340 healthy school children[28]. A clear age-dependent acceptability of the three methods placed OER-nNO as a useful technique to bridge the gap between methods regarding patients 2.5 to 6 yrs of age, who were noncompliant with BH technique. OER-nNO provides an opportunity to measure nNO while achieving plateaus during intermittent soft palate closure and thus providing more certain values compared to measurements
with TB technique. From our data and experience we recommend the following order when attempting measurements in children below 6 yrs of age: OE-R-nNO before TB-nNO.

A number of studies report low nNO levels in patients with an established diagnosis of PCD[3-7] including atypical PCD without typical ultrastructural defects characteristic of PCD[8]. Additionally, nNO has been shown to be consistently low in PCD regardless of the use of different sampling techniques[29]. The reported cut-off levels and corresponding sensitivities/specificities varies between 105 ppb and 94%/88%[3], 187 ppb and 93%/95%[30], and 250 ppb and 97%/90%[5]. Differences may be explained by differences in nasal airflow during sampling and heterogeneity between study populations. In our study we took cut-off levels of 175 (BH-nNO), 242 (OE-R-nNO) and 158 ppb (TB-nNO), and demonstrated comparable sensitivities for all three methods, whereas specificities were only comparable and acceptable for BH-nNO and OE-R-nNO. BH-nNO was superior in subjects exhibiting full cooperation.

Discrimination between PCD and non-PCD was significant for all three methods, both between PCD and HS, and between PCD and non-PCD among the referred patients.

So far, results have been conflicting as to whether PCD and CF can be separated[5;6] or cannot be separated[30] by nNO. In our study, nNO was found to discriminate significantly between PCD and CF irrespective of sampling method. However, overlap occurred unrelated to age, supporting the need to exclude CF before PCD work-up is initiated.
Conclusion

This is the first large study examining nNO as a first line test for PCD in an unselected cohort of referrals of mixed age. We present normal reference values and cut-off values for three different nNO-sampling methods, and suggest an age-adjusted choice of nNO measurement in the following order: BH-nNO > OE-R-nNO > TB-nNO from adulthood through childhood into infancy. nNO discriminated highly significantly between PCD and non-PCD by all three methods, and OE-R-nNO extended the age for soft palate closure measurements. TB-nNO correctly excluded nearly 2/3 of non-PCD referrals below 6 years of age. Future use of normative data from young children and infants may further strengthen the use of TB-nNO. Above-cut-off nNO-values were discovered in confirmed and consecutively referred PCDs, raising the concern that nNO can be misleading in a few patients with true PCD.

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REFERENCES


Fig. 2

OE-R-nNO

nNO concentration (ppb)

HS  PCD  Referrals  Referrals  CF
    NOT PCD  YES PCD

0  200  400  600  800  1000  1200  1400  1600
### Table 1. Characteristics of included subjects.

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>Referrals for PCD work up</th>
<th>PCD patients</th>
<th>CF patients</th>
<th>All subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>57</td>
<td>117</td>
<td>59</td>
<td>49</td>
<td>282</td>
</tr>
<tr>
<td>N (%) males</td>
<td>19 (33.3%)</td>
<td>52 (44.4%)</td>
<td>37 (62.7%)</td>
<td>25 (51.0%)</td>
<td>134 (47.5%)</td>
</tr>
<tr>
<td>Median (range) age in yrs at inclusion</td>
<td>29.5 (3.1 to 63.6)</td>
<td>6.9 (0.0 to 62.4)</td>
<td>17.4 (3.6 to 65.8)</td>
<td>16.4 (0.1 to 50.7)</td>
<td>12.3 (0.0 to 65.8)</td>
</tr>
<tr>
<td>N (%) &lt;16 years</td>
<td>20 (35.1%)</td>
<td>95 (81.2%)</td>
<td>26 (44.1%)</td>
<td>24 (49.0%)</td>
<td>165 (58.5%)</td>
</tr>
</tbody>
</table>

### Table 2. Comparison of mean (SE) nNO levels (ppb) between PCD, CF and HS in the three sampling methods.

<table>
<thead>
<tr>
<th></th>
<th>HS</th>
<th>PCD</th>
<th>CF</th>
<th>(P)-value (PCD vs. HC)</th>
<th>(P)-value (PCD vs. CF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH-nNO</td>
<td>908 (±33); n=49</td>
<td>142 (±42); n=45</td>
<td>416 (±28), n=45</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>OE-R-nNO</td>
<td>788 (±41); n=30</td>
<td>113 (±42); n=35</td>
<td>412 (±76), n=10</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>TB-nNO</td>
<td>534 (±30); n=52</td>
<td>86 (±28); n=54</td>
<td>243 (± 39), n=17</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Subject category</td>
<td>BH-nNO (ppb)</td>
<td>CFT CBF (Hz)</td>
<td>EM defect</td>
<td>Relative with PCD (Y/N)</td>
<td>Neonatal respiratory distress (Y/N/UK)</td>
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<td>------------------------</td>
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<tr>
<td>PCD</td>
<td>1486</td>
<td>Asyn&lt; 8</td>
<td>ODA+IDA</td>
<td>N</td>
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<tr>
<td>PCD</td>
<td>911</td>
<td>Spoke&lt; 8</td>
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<tr>
<td>Ref PCD</td>
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<td>Asyn&lt; 8</td>
<td>ODA</td>
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</table>

PCD: Primary Ciliary Dyskinesia from cross sectional study; Ref PCD: Referrals with confirmed PCD; BH: Breath Hold; TB: Tidal Breathing; CFT: Ciliary Function Test; CBP and CBF Ciliary Beat Pattern and Frequency; Reference for normal CBF (unpublished): 8 to 11 Hz; EM: Electron Microscopy; ODA & IDA: Outer and Inner Dynein Arm; ND: Not done; UK: Unknown; Sol: Solitus; Inv: Inversus; PRMC: Pulmonary Radioaerosol Mucociliary Clearance; BX: Bronchiectasis.