Nasal Nitric Oxide Measurement in Primary Ciliary Dyskinesia A Technical Paper on Standardized Testing Protocols

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Abstract

Nasal nitric oxide concentrations are extremely low in primary ciliary dyskinesia (PCD), and measurement of this nasal gas is recommended as a PCD diagnostic test in cooperative patients aged 5 years and older. However, nasal nitric oxide measurements must be performed with chemiluminescence analyzers using a standardized protocol to ensure proper results, because nasal nitric oxide values can be influenced by various internal and external factors. Repeat nasal nitric oxide testing on separate visits is required to ensure that low diagnostic values are

persistent and consistent with PCD. This technical paper presents the standard operating procedures for nasal nitric oxide measurement used by the PCD Foundation Clinical and Research Centers Network at various specialty centers across North America. Adherence to this document ensures reliable nasal nitric oxide testing and high diagnostic accuracy when employed in a population with appropriate clinical phenotypes for PCD.

Keywords: nasal nitric oxide; primary ciliary dyskinesia; Kartagener syndrome

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Introduction

Nitric oxide (NO) is an important signaling molecule produced throughout the human body. It regulates a diverse range of physiologic and cellular processes in various organs. Within the respiratory tract, normal NO concentrations in the nasal passages are 10–100 times higher than in the lower airways. Although exhaled NO has been used as a clinical marker of lower airway inflammation for decades, NO from the sinonasal system has more recently been recognized as a potential indicator of respiratory disease. Multiple studies demonstrate that nasal nitric oxide (nNO) is much lower in subjects with primary ciliary dyskinesia (PCD) than in healthy control subjects, which provides the foundation for using nNO as a diagnostic test for PCD as recommended in recent clinical practice guidelines from the American Thoracic Society (ATS) (1, 2). Meta-analyses and prospective cohort studies support nNO testing as a safe, noninvasive, feasible, and accurate PCD diagnostic test (3–6). However, nNO testing for PCD diagnosis requires specialized equipment, trained personnel, and standardized operating protocols. These requirements can make access to accurate nNO testing difficult in patients suspected of having PCD. Although ATS/European Respiratory Society (ERS) guidelines on nNO measurement were published in 2005, advances in nNO testing for PCD have shown that some past recommendations require modification (7), because even minor alterations in measurement protocols can significantly affect final nNO values (8–10).

Since 2004, the Genetic Diseases of Mucociliary Clearance Consortium (GDMCC; a multisite research consortium of North American expert PCD centers supported through the National Institutes of Health) and the PCD Foundation (PCDF; a patient advocacy group active in researching PCD and establishing a clinical network of PCD specialty centers across North America) have developed and prospectively employed standardized operating protocols for nNO measurement in patients suspected of having PCD (6, 11). This technical paper on nNO testing addresses the difficulties commonly encountered in nNO measurements for PCD diagnosis. Using the GDMCC and PCDF experiences, we provide guidance on nNO testing procedures and

methodology, as well as assistance on appropriate patient selection, specific testing conditions, and interpretation of nNO results.

Clinical PCD Overview

PCD is a heterogeneous genetic disorder (predominantly autosomal recessive) of motile cilia function resulting in abnormal mucus clearance from the upper and lower airways (12, 13). Altered mucus clearance leads to recurrent otosinopulmonary infections from a very early age. These infections result in chronic progression of both upper and lower respiratory tract disease. Infants with PCD often require weeks of intensive care support for neonatal respiratory distress, and many young children with PCD are affected by profound speech and hearing deficits due to chronic otitis media with ear effusions. Recurrent lower respiratory tract infections, usually diagnosed as recurrent pneumonia or bronchitis, are also common. Most adolescent and all adult patients develop chronic sinusitis and bronchiectasis that can progress to respiratory failure in some cases (11). Key clinical features of PCD in children that help to distinguish PCD from other chronic respiratory issues include the following: 1) neonatal respiratory distress requiring at least 24 hours of supplemental oxygen or positive airway pressure support, despite term birth; 2) year-round sinonasal congestion starting before 6 months of age; 3) year-round wet cough starting before 6 months of age; and 4) an organ laterality defect (situs inversus totalis or situs ambiguus) (14).

NO Overview

Throughout the respiratory tract, NO is produced by a reaction involving substrates (arginine; oxygen; nicotinamide adenine dinucleotide phosphate, reduced), cofactors, and factors catalyzed by NO synthases. Despite early interest in arginine and tetrahydrobiopterin (cofactor) supplementation to influence NO concentrations in a host of various diseases, this therapy has not produced clinically beneficial results or substantial changes in nNO concentrations *in vivo* (15–19). Notwithstanding detailed scientific inquiry, the mechanisms leading to low nNO

concentrations in PCD remain largely unknown (20–22).

Chemiluminescence technology provides for immediate and highly sensitive and specific measurement of NO gas even at extremely low concentrations (parts per billion [ppb]). Through the reaction $[NO + O_3 \rightarrow NO_2 + O_2 + hv]$, NO reacts with ozone to produce nitrogen dioxide, oxygen, and light (hv [wavelength], photons). The intensity of emitted light, which is directly proportional to NO concentration in the sampled gas, is displayed as the concentration of NO (after conversion to suitable units). In clinical settings, the measured concentration of nNO is standardized for the sample gas flow rate and reported as nNO production (nl/min), which is the product of nNO concentration in parts per billion and flow rate in liters per minute (7, 23, 24).

Diagnostic Test Performance of nNO in PCD

nNO concentrations are extremely low in patients with PCD, often less than one-tenth the value of that in healthy control subjects (>300 nl/min in healthy control subjects vs. <77 nl/min in patients with PCD). For most studies of disease control subjects, nNO is usually significantly higher than in patients with PCD (6, 25, 26); however, overlap has been reported for disorders such as cystic fibrosis (CF) (25, 27), diffuse panbronchiolitis (28), and acute viral respiratory infection (29). Thus, CF should be ruled out by sweat testing or cystic fibrosis transmembrane conductance regulator genetic analysis before nNO testing because up to one-third of patients with CF have nNO concentrations below 77 nl/min. Importantly, patients with asthma (30), allergic rhinitis (30), chronic obstructive pulmonary disease (6), immunodeficiency (31, 32), non-CF bronchiectasis (10, 33), and chronic sinusitis without nasal polyposis (34) have higher nNO concentrations than patients with PCD (Figure 1).

Although some studies have demonstrated a distinct difference in nNO concentrations between infants with PCD and healthy infants (<1 –2 yr old), the range between values of these two groups is quite small and easily affected by external factors, including high ambient NO concentrations (29, 35). In general, nNO concentrations in

Figure 1. Nasal nitric oxide (NO) concentrations in subjects with primary ciliary dyskinesia (PCD; 17–180 parts per billion [ppb]) compared with healthy control subjects (543–976 ppb) and subjects with cystic fibrosis (CF; 241–896 ppb), sinusitis (862–3,601 ppb), Young's syndrome (330–1,532 ppb), and bronchiectasis (516–1,098 ppb). Though nasal NO concentration in parts per billion is shown on the y-axis, one should always use nasal NO production values in nanoliters per minute. Because the NO analyzer used to perform these measurements has a flow sampling rate of 0.25 L/min, values below 308 ppb are equivalent to the accepted nasal NO cutoff of 77 nl/min. Only nasal NO values in patients with cystic fibrosis routinely fall below this cutoff value. # $P < 0.0001$. Reprinted by permission from Reference 33.

children younger than 1–2 years old can more frequently overlap with values in healthy control individuals, and thus nNO testing should not be used in this age group until larger validation studies are completed. In children aged 2 years or older but younger than 5 years old, nNO values do not always clearly differentiate between healthy individuals and patients with potential PCD. As children age with accompanying nasal sinus cavity growth, their nNO values also increase, commonly achieving nNO concentrations near adult values after 5 years of age, with smaller increases continuing until age 12 years (6, 36). Thus, if children ages 2 to 5 years undergo nNO testing, often via a tidal breathing method as described below, low values **should not** be used as a stand-alone diagnostic test for PCD and should be repeated after the child turns 5 years of age.

Prospective clinical trials of children aged 5 years and older and adults, consecutively referred for PCD suspicion, have demonstrated high diagnostic accuracy of nNO measurement compared with transmission electron microscopy analysis

and/or PCD genetic testing (3, 6) (Figure 2A). When performed with a standardized protocol using a chemiluminescence NO analyzer, in a patient population with an appropriate clinical phenotype for PCD, and while blowing into a mouth resistor, nNO values less than 77 nl/min are more than 95% sensitive and specific for a diagnosis of PCD on the basis of electron microscopy and/or genetic testing (6) (Figures 2B and 2C). It is essential that patients undergoing nNO testing have a high pretest probability of PCD with the presence of key PCD clinical features. Testing with nNO in a general respiratory population lacking these key clinical features will likely result in worse diagnostic accuracy (37).

Repeat nNO testing on separate visits at least 2 weeks apart is strongly recommended to ensure that low diagnostic values are persistent and not due to occult viral infection or other transient factors. In cases in which low nNO is the only confirmatory PCD diagnostic test (transmission electron microscopy and genetics are negative or inconclusive), serially repeated nNO

measurements over months to years will ensure that concentrations remain low and diagnostic of PCD. In one GDMCC longitudinal cohort, annual nNO testing in 42 patients with PCD showed that nNO values remained steady and below the diagnostic cutoff of 77 nl/min in all but one subject (6). Thus, in patients with PCD, nNO values remain stable in low ranges over time, and normalization of nNO values outside the PCD diagnostic range should prompt clinicians to investigate for other causes of respiratory disease.

nNO Measuring Devices

Only NO analyzers using chemiluminescence technology have been validated in robust clinical PCD studies (3, 11). Smaller patient cohorts have been investigated with less expensive electrochemical NO analyzers; however, these devices are not currently recommended for nNO testing in PCD (38, 39). Although use of electrochemical devices for fraction of exhaled NO measurement in asthma is currently approved by the U.S. Food and Drug Administration and Health Canada, use of these devices for nNO measurement is not similarly approved.

Chemiluminescence NO analyzers are expensive to purchase (\$30,000–\$50,000 USD), and the lack of approval for clinical testing in the United States and Canada prohibits use of these devices outside of research settings in North America. However, these devices are approved for clinical use in Europe. Currently, two companies offer commercial chemiluminescence NO analyzers suitable for clinical testing in North America (CLD88 device from Eco Physics; NOA 280i device from Zysense).

Patient Selection for nNO

Patient selection for nNO testing is critical to maximize diagnostic accuracy (Table 1). Ideal nNO test candidates are cooperative children (usually ≥ 5 yr old) and adults who can blow into a mouth resistor and successfully close their velum to prevent upper airway dilution with lower airway gas. This exhalation against resistance maneuver is quite feasible and is successfully accomplished in more than 90% of subjects aged 5 years and older (3). Less cooperative

Figure 2. (A) Hierarchical summary receiver operating characteristic curve (HSROC) for 12 evaluated studies in a detailed meta-analysis of nasal nitric oxide (nNO) values for diagnosing primary ciliary dyskinesia (PCD) in patients with classic ciliary electron microscopy defects and/or disease-causing variants in a known PCD gene. The summary sensitivity for nNO testing (red square) is 97.6% (range 92.7–99.2) and specificity is 96.0% (range 87.9–98.7). Reprinted from Reference 3. (B) Scatterplot of nNO values (linear scale; nL/min) versus age for University of North Carolina disease control subjects and a subset of subjects with PCD with normal electron micrographs but biallelic mutations in *DNAH11* (solid circles). Individual nNO measurements are shown for disease control subjects: asthma (open circles), cystic fibrosis (open squares), and chronic obstructive pulmonary disease (solid triangles). The dashed line represents the PCD cutoff nNO value of 77 nL/min. Reprinted from Reference 6. (C) Validation nNO data from six PCD specialty sites in the Genetic Disorders of Mucociliary Clearance Consortium. Confirmed cases of PCD had a classic electron microscopy ultrastructural defect and/or biallelic mutations in one known PCD gene. nNO in 155 consecutive individuals investigated for possible PCD was below the 77 nL/min cutoff (dashed line) in 70 of 71 confirmed PCD cases. The open circles represent individuals with PCD confirmed by electron microscopy ultrastructural defect. The solid circles represent individuals with PCD confirmed by genetic testing alone. Reprinted from Reference 6.

children (usually 3–4 yr old) may be able to blow into a child's party favor (Figure 3c) occluded at the distal end to provide adequate airway resistance for velum closure. Occasionally, some subjects aged 5 years or older may not be successful with blowing into any form of resistor, and a tidal breathing maneuver may be necessary in these cases. Some children aged 2–5 years may only be able to perform a tidal breathing technique for nNO measurement, which has been proven to be quite feasible in babies and young children (29), but it has not been shown to have the same diagnostic test characteristics.

Clinicians should have a high clinical suspicion for PCD, with patients manifesting bronchiectasis or at least two key PCD clinical features, before considering nNO diagnostic testing (2). For patients with only one key clinical feature, nNO testing may still be appropriate after ruling out other clinical entities. Unfortunately, nNO concentrations are often in PCD range (<77 nL/min) in healthy children younger than 1–2 years old, presumably because of underdeveloped sinus cavities. Thus, nNO testing for PCD is not currently recommended for patients

younger than 2 years of age. Patients with tracheostomy or significant nasal or facial malformations affecting oral/nasal airflow (unrepaired cleft palate, nasal stenosis/atresia, and severe nasal polyposis) are not ideal candidates either, because nNO concentrations may be secondarily influenced by these issues.

Patients should be in their baseline state of health for at least 2 weeks before nNO testing, because viral respiratory infections can result in extremely low nNO concentrations (and false-positive PCD diagnoses). It is possible that recovery periods after a viral infection may last longer than 2 weeks and that young subjects may acquire another viral organism during this time, necessitating an even longer recovery time before repeat nNO testing (29). However, without solid evidence regarding the time for normal nNO concentration recovery after viral respiratory infection, it seems prudent to wait at least 2 weeks before repeat nNO testing.

Although not definitively studied in large populations, topical nasal medications, nasal lavages, and nasal sprays could affect nNO values and should be avoided for at least several hours before nNO testing (40).

Similarly, nasal instrumentation should be avoided in the days before nNO testing, because denuded or damaged ciliated epithelium from nasal endoscopy or other procedures may affect nNO values. To allow complete recovery of ciliated epithelium, at least 4 weeks of recovery time after any ear or sinonasal surgery should pass before attempting nNO measurement (41). Recent epistaxis can result in very low nNO concentrations because hemoglobin binds NO and acts as a sponge for this molecule in the nasal passages. Tobacco use may artificially increase nNO concentrations and should also be avoided for several hours before nNO testing (42). Although consumption of foods rich in nitrates could conceivably alter nNO concentrations (43), there is no evidence to support requiring patients to be in a fasting state for nNO testing.

Personnel and Training

Technicians operating chemiluminescence devices should be trained in approved operating protocols (see Appendixes E2 and E3 in the online supplement). Hands-on

Table 1. Causes of false-positive and false-negative results in nasal nitric oxide testing

False-positive test (low nNO values)

- Acute viral respiratory infection (29)
- Cystic fibrosis (25, 27)
- Diffuse panbronchiolitis (28)
- Poor technique (leaking around resistor or nasal catheter)
- Severe nasal obstruction
- Fresh blood in the nasal cavity
- Recent sinonasal surgery or instrumentation
- Naso-oro-facial malformations preventing closure of the soft palate
- Obstructed sampling line*

False-negative values (normal nNO values)

- High ambient NO concentrations
- PCD from *RSPH1* gene mutations[†] (53)
- GI source (burping during procedure)
- Obstructed sampling line*

Definition of abbreviations: GI=gastrointestinal; nNO = nasal nitric oxide; NO = nitric oxide; PCD = primary ciliary dyskinesia.

*Obstruction of the sampling line is more likely to result in low nNO values, and this error is avoided by confirming stable sampling flow rates before and after each patient tested.

[†]It is estimated that less than 2% of all PCD is caused by *RSPH1* mutations, but not all patients with PCD through *RSPH1* mutations have normal nNO concentrations. Other PCD cases with normal nNO values have been intermittently reported with various other genetic and electron microscopy defects, but *RSPH1* mutations have been repeatedly linked to normal nNO concentrations in PCD.

training sessions are available through the PCDF and at the annual PCD On the Move Conference sponsored by the PCDF.

Training provided by chemiluminescence device manufacturers may not be adequate for clinical diagnostic testing and may not adhere to GDMCC or PCDF protocols. Annual competency training of all personnel operating chemiluminescence devices is also recommended. At least two fully trained technicians are recommended at a clinical site so that employment turnover does not affect testing capability. Responsibility for device maintenance and scheduled calibrations should rest with trained personnel.

Pretest Patient Check

Nasal examination using a standard otoscope is recommended at the time of nNO measurement because severe nasal

obstruction, nasal malformation, or fresh blood in the nares can result in artificially low nNO concentrations. Patients with nasal blockage, acute sinusitis, recent or acute nosebleed, or acute viral respiratory infection should not be tested. The technician should ask patients to blow their nose using a tissue and breathe through each nostril to make sure that they are open and clear. The patient should be seated away from the instrument exhaust because the exhausted air may not be completely scrubbed of NO, which could potentially influence nNO measurements if inhaled by a patient.

nNO Testing

Permanent Equipment

In addition to the NO analyzer, sites should have an external flowmeter capable of measuring the sampling flow rate of the NO analyzer within a desired range of approximately 200–600 ml/min (Table 2). In the Eco Physics CLD88 analyzer, with a

no. 3 resistor in line, the flow rate should be steady at 330 ml/min ($\pm 10\%$). Different sampling line resistors, which are provided by the manufacturer, will result in different flow rates requiring recalibration of the instrument. Similarly, the sampling flow rate in the Zysense NOA 280i analyzer should also be steady and near 300 ml/min ($\pm 10\%$). Although recommended ATS/ERS flow sampling rates are 0.25–3 L/min, rates greater than 0.5 L/min (500 ml/min) will likely cause discomfort in smaller children and possibly deplete nNO stores faster than production, resulting in false-positive PCD diagnoses (7, 23, 24). Because significant changes in measured nNO values occur over a range of different transnasal flow rates (44), it is important that current nNO devices use similar sampling airflow rates to allow comparison of nNO values across various devices and diagnostic centers. Thus, reports of nNO values should include the flow sampling rate used for the measurement.

Figure 3. (A) Nasal catheters with surrounding nasal sponge olives in three different sizes. Note the built-in air filter at the opposite end in each of these disposable catheters. The neonatal-size catheter is not shown here. Manufactured by DirectMed. (B) Disposable cardboard tube resistor. Patients securely seal their lips around the open end and gently blow in a sustained and prolonged manner into this resistor to achieve velum closure and to prevent dilution of sampled nasal gas with lower airway flow. Note the pinhole opening (white arrowhead) in the red resistor cap, which provides approximately 3–5 cm H₂O of airway resistance. Manufactured by DirectMed. (C) A 3-year-old child performing an exhalation against resistance nasal nitric oxide measurement using a child's party favor resistor. Note the tape occluding the distal end of the party favor and the sustained exhalation maneuver keeping the party favor in an extended position long enough to achieve a plateau value. (D) A 7-year-old child performing an exhalation against resistance nasal nitric oxide measurement using a cardboard tube mouth resistor. The lips are securely tightened around the resistor to avoid leaking.

Table 2. Required equipment to perform nasal nitric oxide testing for primary ciliary dyskinesia diagnosis

Equipment	Estimated Cost (USD)	Required Maintenance Schedule and Cost
Chemiluminescence NO analyzer	\$30,000–\$50,000	Annual maintenance: \$1,000–\$2,500/yr
nNO scrubber for ambient NO washout	\$3,000–\$5,000	Filter cartridge change annually or when filtered air is no longer <3 ppb: \$150 per filter
External flowmeter	\$300–\$600	None
NO device calibration gas	\$300–\$800 per tank	Replace tank per supplier expiration or when tank pressure falls below 100 kPa (15 psi)
Nasal catheters in various sizes	\$5–\$10 per patient	Disposable
Cardboard resistors and party favors	\$3–\$5 per patient	Disposable

Definition of abbreviations: nNO = nasal nitric oxide; NO = nitric oxide; ppb = parts per billion.

Under certain circumstances, such as a small testing room with poor ventilation, ambient NO may accumulate and become significantly elevated. When this occurs, operators should use NO-free air from a certified NO-free cylinder, central NO-free air wall outlet, or a stand-alone NO scrubber so that inflow air into one of the nostrils is free of NO during a test (see PRETEST EQUIPMENT CHECK section). If ambient NO concentrations are routinely greater than 50 ppb, such as at an institution located in a highly air-polluted area, more permanent options to decrease ambient NO in the testing room should be explored. However, if the measured patient nNO values are substantially above the ambient NO values (e.g., more than five times higher, as they would likely be in healthy individuals without PCD), the test can be accepted as valid while making note of the higher ambient NO concentration. A computer, either integrated into the system or set up as a standalone connected to the NO analyzer, controls the system's operation, including acquisition, analyses, storage, archiving, and, if desired, printing of graphs and data.

Disposable Equipment

Appropriate-sized disposable nasal probes with spongy olives for nasal insertion are required. Preferably, these disposable probes should have built-in filters to avoid nasal debris being sucked into the sampling line. If built-in filters are lacking, operators should use inline filters provided by the device manufacturer. Most commonly, four sizes of

nasal probes are available for commercial purchase: neonatal, small adult (for children 2–5 yr old), medium adult (for children >5 yr old and petite adults), and large adult (Figure 3A; neonatal probe not shown).

Disposable mouth resistors are also required for nNO testing. These should provide at least 3–5 cm of water pressure resistance, which is adequate to close the velum and prevent upper airway air dilution with lower airway air. Inexpensive cardboard tubes with a pin-sized hole in a plastic endcap are ideal for this purpose (Figure 3B). For younger children who may have difficulties with cardboard resistors, a child's party favor, tightly occluded at the distal end with lightweight adhesive tape, is also acceptable as a resistor (Figure 3C). Blowing against a cardboard resistor will ensure closure of the palate and eliminate contamination of nNO by oral and exhaled gases during measurement.

Pretest Equipment Check

Using the external flowmeter with a protective filter in place, technicians should measure the flow rate in the sampling line before and after each patient is tested. This value should be recorded for each patient because major changes in this flow rate (more than $\pm 10\%$ from usual flow) indicate a leak or obstruction in the sampling line and may greatly alter final nNO values. If the flow rate is outside the usual range, then technicians should recalibrate the device, and if the flow is still outside the usual range, then the sampling line or the inline resistor

should be replaced and the device recalibrated. This quality assurance step will verify the flow sampling rate as steady before and after each patient is tested and avoid false-positive and false-negative results that can occur if flow rates change from the usual value. NO devices may not always alarm or automatically notify the technician of flow sampling changes.

The technician should check and record the ambient concentration of NO in the room for each patient tested. Though there is no solid evidence for ambient NO cutoff values, when ambient NO concentrations are greater than 50 ppb for a resistor breathing test or greater than 10 ppb for a tidal breathing test, an NO washout is recommended before and during the test. To accomplish this washout, the technician places the nasal catheter into the desired naris and, using a source of NO-free air or an NO scrubber device, allows NO-free air to gently blow against the nonoccluded naris for 1 minute as the patient breathes via the nonoccluded naris (Figure 4). After 1 minute of breathing NO-free air, the nNO measurement may begin. Throughout the measurement, the technician continues to gently blow NO-free air (from ~2-cm distance so as not to increase air pressure in the nose) toward the nonoccluded naris. Then, the technician changes the probe to the contralateral naris and repeats the identical ambient washout and measurement steps. All nNO measurements requiring an ambient NO washout should ideally be repeated on a separate occasion to ensure that values are stable, repeatable, and valid.

If ambient NO values are within normal limits, the technician places the appropriate-sized nasal catheter with spongy olive into one naris to a depth of 0.25 to 0.375 inch into the nostril. The olive should form an airtight seal and rest securely in place. If technicians see rhythmic movement of the olive in and out of the nostril with each breath, then the fit is too loose, and a larger olive may be necessary. Large-sized olive sponges may be trimmed with scissors to the appropriate fit, as long as the airtight seal is maintained in the nostril.

Depending on the patient's age and degree of cooperation, the technician should demonstrate the exhalation technique that he or she thinks will be most successful. Cooperative children aged 5 years or older and adults should be able to blow into a cardboard tube resistor (Figure 3D). If

Figure 4. Ambient nitric oxide (NO) washout technique. When the ambient NO value is greater than 10 ppb for a tidal breathing test or greater than 50 ppb for a resistor breathing test, an NO washout should be performed before and during the test. The technician places the nasal catheter into the desired naris and, using the NO scrubber device, allows NO-free air to blow into the nonoccluded nostril for 1 minute as the patient continues to breathe via their nonoccluded nostril. This source of NO-free air is continually blown into the nonoccluded nostril until testing is complete.

exhalation against a cardboard mouth resistor is not possible, then technicians may try a child's party favor as a resistor or choose to use a tidal breathing method.

Testing Maneuvers

Exhalation against mouth resistor and party favor technique. Testing should be performed in a software mode that allows the technician to view the nNO concentration curve in real time and then manually determine the actual plateau value upon test completion. Software with automated plateau detection should be avoided because significant differences in plateau values have been demonstrated with automatic versus manual plateau measurements (9).

After explaining or demonstrating the procedure, the technician attaches the flow sampling line to the nasal probe with attached filter, starts the test on the NO device computer, and inserts the probe into the patient's nostril. The patient is asked to hold it slack with the hand on the side of the inserted probe (ipsilateral) so that the probe

is not accidentally loosened or pulled out of the naris. With the other hand, the patient holds the mouth resistor. The patient is asked to inhale to near-total lung capacity and then place the resistor into their mouth, sealing it well with tight lips. With the cheeks slightly puffed, the patient blows in a

steady, low-flow, prolonged manner until he or she is out of breath or until directed to stop by the technician. The technician monitors the nNO curve for an acceptable plateau of at least 3 seconds on the computer screen; it may take up to 30 seconds of exhalation time to achieve a proper plateau. It is not necessary to blow hard (as with forced maneuvers in spirometry), but the technician must check the end of the resistor to make sure there is air flowing through the small hole in the resistor and not around the resistor due to leaking from the patient's lips. For younger children, the technician should hold the sampling line slack during exhalation maneuvers. If blowing into a party favor resistor, patients should continue a prolonged exhalation maneuver that keeps the favor in an extended position until an acceptable plateau is obtained (Figure 3C).

After one successful plateau value, recovery from the prolonged exhalation, and around 30 seconds of rest between trials, the patient then performs a second maneuver for repeat measurement in the same naris. This may require several attempts until two successful trials are achieved in the first nostril (Figure 5). Both plateau values should be within 10% of each other. Further acceptability standards for nNO measurements are discussed in detail below. The procedure is then repeated in the contralateral nostril for two further plateau measurements in the same fashion.

Upon test completion, the technician should again measure and record the sampling flow for consistency. If the flow is

Figure 5. Nasal nitric oxide (NO) curve of a patient exhaling into a cardboard resistor. Note that the first plateau may seem acceptable, but the following two plateaus are more than 10% higher, reflecting improved exhalation technique upon repeat maneuvers. Thus, the last two plateau values would be used to calculate the nasal NO in this case, whereas the first plateau would be discarded. ppb = parts per billion.

changed by more than $\pm 10\%$ from the pretest flow rate, then the sampling line or an inline resistor has likely become obstructed by nasal secretions, and the nNO measurement should be discarded. The sampling line or the inline resistor will likely need to be changed to fix this issue.

Under certain conditions in very young patients or even adults, the mouth resistor technique may not give acceptable results. In such cases, a tidal volume technique, which is much easier to accomplish, should be used. Tidal nNO values are roughly two-thirds of a resistance value, and if poor technique is suspected in exhalation against resistance testing, a tidal breathing value of two-thirds the resistor value can help verify adequate resistor technique (*see TIDAL BREATHING nNO MEASUREMENTS* section).

Breath-hold measurement. In addition to exhalation against resistance techniques, breath-hold maneuvers are also an appropriate method of achieving velum closure during nNO measurements, and studies have shown that breath-hold maneuvers are equivalent to exhalation against resistance in discriminating subjects with PCD from subjects without PCD through nNO testing (10, 45–47). In fact, breath holding is one of the reported methods in early nNO studies (22). To achieve a proper breath hold, subjects are instructed to inhale to total lung capacity and then hold their breath, thereby closing their velum until a 3- to 10-second nNO plateau is produced (47).

Despite the extensive use and validity of breath-hold maneuvers for nNO measurement, ATS/ERS guidelines recommend exhalation against resistance as the preferred method of velum closure in nNO testing, with breath hold as an alternate maneuver (7). Similarly, the PCDF recommends exhalation against resistance as the preferred maneuver for nNO measurement for several reasons. First, short-term reproducibility is superior in exhalation against resistance versus breath-hold maneuvers (48). Second, complete velum closure during breath-hold maneuvers requires good subject cooperation, which is difficult with children and even with some adults (45). In addition, mean nNO values by breath-hold maneuver are lower and have a greater standard deviation than exhalation against resistance maneuvers, pointing to possible incomplete velum closure with breath-hold technique in some patients (10, 48). Furthermore, gas

mixing occurs *in vivo* at the intake flow rate of the NO instrument; within 2–5 seconds, gas is entrained from any low-resistance luminal orifice connected to the sampling nostril (22). Because pulmonary and pharyngeal NO concentrations are lower than nNO concentrations, this entrainment from subnasopharyngeal cavities will dilute the nasal sample, decreasing the signal-to-noise ratio in identifying subjects with PCD. This will inevitably make the breath-hold test less specific than exhalation against resistance. Also, 5 cm of water back-pressure, achieved through exhalation against a resistor, has been unequivocally demonstrated to close the velum, making the measurement of nasal and sinus air uniform and undiluted (49). This obviates the problem of having a low signal-to-noise ratio. Moreover, a substantial amount of work has already been done in subjects with PCD and control subjects using the specific method of exhalation against resistance (6, 25, 31, 32, 50–53). The recommendations here are based on data from hundreds of subjects using this specific exhalation against resistance technique. In the future, if a breath-hold technique is to be officially recommended by the PCDF, large-scale validation studies will need to be repeated with breath-hold maneuvers. Thus, the PCDF standard operating protocols for nNO measurement recommend exhalation against a resistor as the preferred maneuver to achieve velum closure in nNO measurement (Appendixes E2 and E3). Consideration could be given to future studies in which data from control subjects

and subjects with PCD are collected using a breath-hold technique.

Tidal breathing nNO measurements.

Uncooperative patients (generally 2–5 yr old) who cannot blow into a mouth resistor or party favor may perform a tidal breathing nNO test. The test can also be performed in older children and even adults if they are unable to perform a mouth resistor breathing test. For a tidal breathing test, the small child can sit in a parent's lap, with the parent gently restraining the arms to prevent pulling on the nasal probe. The parent or technician will often need to hold the nasal probe in place as well. Allow the patient to breathe quietly, and remind the patient to keep their mouth wide open during the test, because closed-mouth techniques can result in significant nNO differences. Distraction techniques with movies, mobile phones, books, or other devices are quite helpful in these cases. Operators should record at least 60 seconds of nNO signal in the first naris, looking for at least five maximal and repeatable tidal peaks within 10% of each other (Figure 6). Shorter recording times may lead to inaccurate results (Figure 7). Uncooperative children may take longer to provide reproducible tidal peaks.

If a child is distressed because of sampling line suction (may be uncomfortable but should not be painful) or the nasal probe becomes plugged with mucus, reposition and/or change the probe in the naris and ask the patient to clear their nose with a tissue. If nasal probes are repeatedly plugging with secretions, then the child is likely not in a baseline state of health, and testing should be rescheduled for

Figure 6. Nasal nitric oxide (NO) curve of a cooperative 4-year-old child performing nasal NO measurement with a tidal breathing technique. Note the reproducibility and stability of numerous tidal peaks just above 140 parts per billion (ppb; white arrows).

Figure 7. Nasal nitric oxide (NO) tracing of a 3-year-old child. For the first 40 seconds, the reproducible and maximal tidal peaks fall near 250 parts per billion (ppb; black arrows). However, after 60 seconds, the maximum reproducible peaks are higher and fall near 325–350 ppb (white arrows). Thus, tidal breathing tests should record for at least 60 seconds to ensure that reproducible, maximal tidal peaks are detected. One peak is clearly an outlier (gray arrow) because it is not reproducible and more than 10% above the other tidal peaks.

another day. Once operators have acquired five maximal and reproducible tidal peaks, they can withdraw the probe from the nostril. The same measurement is then performed in the contralateral naris. As with mouth resistor technique, the technician should again measure and record the flow rate in the sampling line for consistency.

Acceptability of Results

Using plateau measurement software included with NO devices, technicians should choose two acceptable mean plateau nNO values (obtained with exhalation against resistance) in each naris. Acceptable nNO plateaus should be 3 seconds or longer in duration and have less than 5% variation within the measured range. The variation percentage is automatically displayed by the program. Technicians should also use maximum attainable mean plateau values. Plateau values within the same naris should be within 10% of each other, whereas values between the right and left nares should ideally be similar, but larger variation may be present due to unilateral differences in nasal airflow (due to polyps, nasal obstruction, septum malformation, etc.). Testing in each naris should be repeated at least twice in the same session if a greater than 10% difference exists between the right and left nares, with the maximum repeatable values in each naris reported even if this difference remains present. In patients with very low nNO values (as in those with PCD), obtaining values strictly within 10% of each

other may be difficult, but this should not preclude using these values if all of them fall well below the cutoff of 77 nl/min. Often, the first exhalation maneuver produces values more than 10% below subsequent exhalation trials as patients familiarize themselves with the exhalation technique (Figure 5).

Acceptable tidal breathing technique requires the technician to choose five maximal and reproducible nNO tidal peaks in each naris; they do not have to be

consecutive. The maximum value for each peak is chosen, as opposed to the mean value in plateau measurements. From within a single naris, the five tidal peaks must fall within 10% of each other. In case of intermittent nasal obstruction, which is not always easy to detect in younger children, there may be one or several very high “outlier” nNO peaks surrounding the lower, more reproducible tidal peaks. Sometimes, the lower peaks may occur below the diagnostic cutoff, whereas the higher peaks may fall above this cutoff. If operators cannot definitively tell which set of peaks is actually the most reproducible, then the results are invalid, and the test should be repeated at another visit.

Technicians should allow the device to record for at least 60 seconds to ensure that the chosen tidal peaks indeed comprise the highest reproducible set of peaks in that naris (Figure 8). Uncooperative or crying children may spontaneously perform breath-holding maneuvers resulting in plateau values mixed with tidal peaks. If this occurs, plateau values may be chosen as one of the five tidal peaks if they are within 10% of the surrounding tidal peaks, but operators should exercise caution with values obtained from crying children and repeat the test at another time when the child is calm (Figure 9). As in exhalation against a mouth resistor, tidal breathing nNO values between the right and left nares should ideally be

Figure 8. Nasal nitric oxide (NO) tracing of a 3-year-old child performing a tidal breathing maneuver. Although there is a reproducible set of tidal peaks near 250 parts per billion (ppb; black arrows), there are higher reproducible tidal peaks near 400 ppb (white arrows). These higher peaks (white arrows) should be chosen as the maximal values to use for final nasal NO calculations.

Figure 9. Nasal nitric oxide (NO) tracing from an uncooperative, crying child performing a tidal breathing maneuver. With spontaneous, prolonged exhalation maneuvers during crying, this child produces plateau values that can also be used as tidal peaks (peak numbers 2 and 4). ppb = parts per billion.

similar, and greater than 10% internaris variation should prompt repeat testing bilaterally during the same session. All nNO measurements requiring an ambient NO washout, whether performed by exhalation into a mouth resistor, exhalation into a party favor, or tidal breathing technique, should be repeated on a separate occasion to ensure that values are valid.

Calculations

After choosing acceptable plateau or peak tidal points, the technician analyzes them with the appropriate software. For the final calculation with mouth resistor testing, the two acceptable concentration values within each naris are averaged. Calculations for tidal breathing are performed in a similar manner, using the average of all five tidal peaks in each naris. Then, concentrations in the right and left nares are averaged together, yielding a final mean concentration in parts per billion. Subsequently, the final averaged value in parts per billion is “normalized” by converting it into nanoliters per minute as follows:

Mean concentration of right and left nares together (ppb) $\times$ Flow sampling rate (L/min) = Final nNO value (nl/min): e.g., 1,000 ppb $\times$ 0.33 L/min = 330 nl/min

Simply providing the measured nNO concentrations in parts per billion is not adequate for PCD diagnosis, because

different flow sampling rates can greatly alter final nNO values (24, 54). If measured flow rates before and after nNO testing are stable ($\pm 10\%$) on a single device, one standard flow rate could be used for all calculations to avoid mathematical error. An autocalculating spreadsheet is similarly recommended to avoid computational errors.

Interpretation of Results

nNO values in healthy control subjects aged 5 years and older measured by exhalation against mouth resistance are typically greater than 250–300 nl/min, whereas values less than 77 nl/min (after ruling out CF) are highly sensitive and specific for a diagnosis of PCD (3, 6). However, there are rare, mild forms of PCD (e.g., those caused by variants in *RSPH1* [radial spoke head component 1]) resulting in nNO concentrations greater than 77 nl/min (53). Though most of these mild cases produce nNO concentrations in the lower range of normal (78–125 nl/min), they can occasionally result in very normal nNO values (>250 nl/min). Thus, a resistor value much higher than 77 nl/min makes PCD very unlikely, though still possible. Patients with clinical phenotypes that are highly suggestive of PCD should proceed to further PCD diagnostic investigations (electron microscopy and/or genetic testing), regardless of nNO values, especially after other similar clinical entities are ruled out, such as CF, immunodeficiency, and chronic aspiration.

Low nNO values should always be verified on a separate occasion at least 2 weeks from the initial low value.

Although definitive PCD diagnostic cutoffs for tidal breathing nNO are currently lacking, a tidal breathing nNO value well above 77 nl/min is also reassuring that PCD is unlikely; however, it is still possible in isolated cases. Because nNO concentrations are age dependent until roughly 12 years old, values less than 77 nl/min in tidal breathing testing are sometimes seen in preschool healthy control subjects (55, 56). Several research publications have suggested that tidal nNO values less than 37–50 nl/min are consistent with PCD, but these populations sometimes included subjects older than the 2–5-year age range in which this technique and cutoff value are used, and both remain to be validated (55–57). Thus, definitive nNO diagnostic cutoffs are not strictly delineated for preschool children, but normal tidal nNO values (>77 nl/min and possibly >37 –50 nl/min) are reassuring for not having PCD, whereas low nNO concentrations may be, from a young age, occult respiratory viral infection, CF, or PCD. Low nNO values measured via tidal breathing **should not** be used as a stand-alone diagnostic test for PCD in preschool children, and all tidal breathing nNO tests should be serially repeated to make sure results remain consistent over time.

Device Maintenance

Chemiluminescence NO devices should be maintained and calibrated to manufacturer specifications, including replacement of degradable components (internal scrubbers, valves, etc.) at least once every 1–2 years. Device calibration should be performed at least once monthly when using these devices for PCD testing. Because plastic sampling lines degrade over time, measurement of flow sampling rates should be performed before and after each subject is tested, and the sampling line or inline resistor, if applicable, should be changed when these rates start to vary significantly. Any change of sampling line, inline resistor, or other critical components should also be followed by device calibration. In general, a low-concentration NO gas supply (1–5 ppb) is required for calibration, and this can be specially ordered from most medical gas suppliers. Significant error messages from

NO devices should result in recalibration after correcting errors and restarting the device.

Technicians should also establish logbooks of device maintenance, repairs, and biologic controls. By knowing their own usual nNO concentrations, technicians can check themselves after calibration to confirm that their “biologic control” concentrations are stable and that the device is functioning properly. Technicians can also investigate aberrant nNO results (e.g., normal nNO values in a patient with situs inversus totalis, significant neonatal respiratory distress,

and chronic wet cough) by testing for stability in their previously known biologic control nNO values.

Conclusions

nNO measurement is a quick, noninvasive, and accurate test for diagnosing PCD. Though diagnostic cutoff values have not been strictly established in preschool subjects performing tidal breathing maneuvers, older children and adults can benefit from this highly feasible test.

Obtained through standard operating procedures as outlined here, nNO values are extremely informative in patients with suspected PCD, but current NO chemiluminescence devices lack regulatory approval for clinical use and must be operated by trained and experienced personnel. Despite these shortcomings, nNO measurement is an established diagnostic test for PCD, and proper device maintenance and operation and interpretation of results are essential to improving diagnostic outcomes in PCD. ■

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