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ABSTRACT

Background: Nasal nitric oxide (nNO) is decreased in patients with primary ciliary dyskinesia (PCD) as compared to healthy subjects (HS). nNO measurement has been proposed as a screening tool for PCD, but NO analyzers are not widely available and reference values are lacking.

Objectives: to determine the normal values of nNO in adults using a widely-used hand-held NO analyzer, and explore correlations with ambient NO, age, gender, weight, height, body mass index (BMI), and time of measurements.

Methods: in a pilot phase, 25 adult HS had nNO sampled during breath hold (BH), tidal mouth breathing through a fixed resistance (straw, TB-S) and tidal breathing through a continuous positive airway pressure apparatus (TB-CPAP). TB-S was the most reproducible and comfortable sampling technique and was chosen to measure nNO in 200 adult HS of both genders aged 20 to 80 years, and in 7 adults with PCD.

Results: In HS, mean nNO adjusted for ambient NO was 517 ± 226 ppb and was negatively correlated with age ($\beta = -3.76$, $p = 10^{-4}$). No correlation was found with gender, weight, height, BMI, month of the year, and time of the day. The lower limit of normal nNO (2.5th centile) decreased from 201 ppb at age 20 to 46 ppb at age 80. 6 out of 7 PCD patients had nNO below lower limit of normal.

Conclusions: nNO values significantly decrease with age in adult HS. Reference values are available to use a handheld nNO analyzer as a screening tool for PCD in adults.

Key words: Kartagener syndrome, ciliary motility disorders, primary ciliary dyskinesia, nitric oxide, nose, healthy volunteers, adult

INTRODUCTION

Primary ciliary dyskinesia (PCD) is a rare autosomal recessive disorder characterized by dysfunction of motile cilia of the respiratory epithelium resulting in impaired mucociliary clearance. The main clinical manifestations of PCD are recurrent sinopulmonary infections leading to the development of bronchiectasis, and eventually chronic respiratory insufficiency. The diagnosis of PCD requires complex analyses of ciliary ultrastructure, beating frequency and pattern, and/or genetic studies, which are available in only a few specialised centers. For this reason, a simple screening tool is highly desirable.

A significant decrease of nasal nitric oxide (nNO) has been observed in patients with PCD compared with healthy subjects (HS) (1–14) and several studies have suggested that nNO measurement could be used as a screening tool for PCD (1–4,6–13,15). However, several methods have been reported for nNO sampling and measurement, and no standardized protocol is currently available. Consequently, reference values for nNO are lacking, thus preventing to accurately identify patients with abnormally low nNO.

The NIOX MINO is a hand-held NO analyzer, which is widely used by respiratory physicians to measure exhaled NO in asthmatics. By using a nasal adapter, the NIOX MINO can also be used to measure nNO. This device demonstrated a high reproducibility and a good correlation with other techniques (11,13,16–19) that are bulkier and more expensive. It could therefore be a screening tool for PCD.

This study aimed at 5 objectives: 1) determine whether nNO can be reliably measured with the NIOX MINO, 2) identify the most reproductible and comfortable breathing technique to measure nNO, 3) explore possible correlations between nNO and ambient NO, age groups, gender, weight, height, body mass index (BMI), month of the year and time of the day, 4) determine normal nNO values in adult HS, 5) compare nNO values in HS and patients with PCD.

METHODS

Subjects

200 HS were recruited prospectively between December 2013 and October 2014 by word of mouth and through flyers distributed to the personnel of the Lausanne University Hospital. The subjects were stratified by gender and age to include 100 men and 100 women distributed equally into five 10-year age groups (20-30, 30-40, 40-50, 50-60, and > 60 years). Active smokers and subjects with the

following conditions were excluded: asthma, acute or chronic sinusitis, nasal polyps, sinus surgery, upper airway infection in the last 3 weeks, use of nasal corticosteroids in the last 3 weeks, nasal regurgitation during swallowing, cleft palate, uvulopharyngopalatoplasty, neuromuscular disorder, and chronic respiratory disease including cystic fibrosis and PCD. Patency of both nostrils and symmetrical motion of the soft palate were assessed before measurements. A group of 7 patients with PCD were also included for comparison with HS. All subjects gave written consent. The study was approved by the local Ethics Committee and recorded on ClinicalTrials.gov (Identifier NCT02133547).

Material

Nasal NO measurements were performed with the NIOX MINO analyzer (Aerocrine, Solna, Sweden), a hand-held device for measurement of exhaled NO, which can also be used to determine nNO. The analyzer is based on an electrochemical sensor technology. It covers a range of values between 5 and 1700 parts per billion (ppb) and does not require calibration. To measure nNO, a disposable standard tubing is connected to the analyzer. This tubing is terminated by a nasal olive used to occlude the nostril. During sampling, ambient air is aspirated at a constant flow rate into one nostril, circulates around the posterior nasal septum, and enters through the other nostril into the sampling tubing then the analyzer. The aspiration flow rate may be set at 2 or 5 ml/sec, resulting in sampling times of 120 or 45 seconds, respectively.

Measurement of nNO

An airflow rate between 0.25 l/min (4.2 ml/s) and 3 l/min (50 ml/s) has been recommended for nNO measurements (20). We used a rate of 5 ml/s, which has been used in previous studies (9,11,13). One methodological issue in nNO measurement is to avoid contamination of nasal air sample by exhaled alveolar air. Various techniques have been used previously for this purpose (9,11,13,14,21–23), and nNO appeared consistently low in PCD with all them (9,23). In a pilot phase of the present study, we compared 3 different breathing techniques for this purpose on the first recruited 25 subjects: 1) breath holding (BH), 2) tidal mouth breathing through a fixed resistance (straw) (TB-S), and 3) tidal breathing through a continuous positive airway pressure apparatus (TB-CPAP). Only the first 2 techniques have been previously described (9,11,13–15,21–23). For BH, the subject was asked to hold his breath during nNO sampling, i.e. 45 seconds. For TB-S, the subject was instructed to breathe spontaneously through the mouth with a straw in order to create a small positive pressure in the oropharynx and allow soft palate closure during exhalation. For TB-CPAP, the subject was asked to breathe spontaneously

through a mouthpiece connected to a continuous positive airway pressure apparatus set at a pressure of 4 cm H2O. This technique aimed at providing soft palate closure throughout the whole respiratory cycle during quiet spontaneous breathing. Each subject performed 3 measurements of each of the 3 methods, and was asked to rank each method from the most (3 points) to the less (1 point) comfortable. The method with the highest reproducibility and highest comfort score was chosen for determination of normal nNO values in total of 200 HS, including the 25 HS of the pilot phase. All measures were made in triplicates. Ambient NO was measured systematically. For comparison, nNO was also measured in 7 patients with PCD.

Statistics

The nNO value of each subject was expressed as the average of 3 consecutive measures. Data are presented as mean ± standard deviation. Test-retest reliability was assessed with intraclass correlation coefficient (ICC). Linear regression was used to explore associations between nNO and respectively ambient NO, age, gender, weight, height, BMI, month of the year and time of the day. A p-value <0.05 was considered significant. Calculations were performed with Stata.

RESULTS

Study population

The study population consisted of 200 HS including 100 women and 100 men distributed equally into five age groups of 10 years (20-30, 30-40, 40-50, 50-60, > 60 years). The mean age was 44.3 years, the mean weight 71.0 kg, the mean height 169 cm, the mean BMI 24.7 kg/m2, and the mean ambient NO 14 ppb. 7 adult HS aged 70 and over were included, the eldest was a 80 years old man. 17 subjects were excluded: 4 for asthma, 2 for chronic sinusitis, 6 for active smoking, 3 for nasal polyps and 2 for sinus surgery. A group of 7 patients with PCD were also included for comparison with HS. Their characteristics were: mean age 34.3 years, mean weight 65.6 kg, mean height 174 cm and mean BMI 21.4 kg/m2.

Choice of sampling method

The results of the pilot phase used to determine the most reproducible and most confortable sampling method in 25 HS are shown in table 1. The intraclass correlation coefficient (ICC) was highest for TB-S. The comfort score was also highest for TB-S. This method was thus chosen to determine normal values of nNO in the whole study population.

Determination of normal values of nNO

The main average data are shown in table 2. The mean ambient NO was 14 ± 10 ppb. A significant correlation was observed between nNO and ambiant NO ($\beta = 4.07$, $p = 0.013$). To avoid any bias, ambient NO values were subtracted from the raw nNO values to obtain adjusted nNO values (nNO_{adj}). After this substraction, the mean nNO_{adj} in the study population was 517 ± 226 ppb. ICC between the three measurements was 0.85.

By linear regression, age was significantly associated with nNO_{adj} ($\beta = -3.76$, $p = 10^{-4}$, $r^2 = 0.06$). This means that for each increase in age of 1 year, nNO_{adj} decreased of about 4 ppb. No significant correlation was found with gender ($p = 0.47$), weight ($p = 0.16$), height ($p = 0.58$), BMI ($p = 0.05$) and hour of the day ($p = 0.46$). Taking January as reference, no correlation was found between month of the year and nNO_{adj}, except for May ($\beta = -221$, $p = 0.03$), but only 9 subjects were sampled during this month.

As age was associated with nNO_{adj}, normal values according to age were calculated by increments of 1 year and expressed for the percentiles 2.5, 5, 25, 50, 75, 95 and 97.5 (table 3 and figure 1). The 2.5 and 97.5 percentiles were considered as the lower and upper limits of normal, respectively.

The values of nNO_{adj} in adult HS were compared with those of patients with PCD. A markedly reduced nNO_{adj} was observed in patients with PCD (figure 2). On average, nNO_{adj} was 60 ± 54 ppb in this group. One 34 year-old female with PCD had a nNO_{adj} value of 178 ppb, which was slightly above the lower limit of normal (percentile 2.5, 163 ppb), but still below percentile 5 (218 ppb). All other patients with PCD had nNO values below the lower limit of normal.

DISCUSSION

To our knowledge, this study is the first to determine normal values of nNO in adults, using a large cohort of HS of both genders and all age groups, and analyzing various parameters that can influence the values of nNO. We found that TB-S is the most reproducible and comfortable breathing techniques for nNO sampling. We demonstrated that nNO was significantly and negatively correlated with age, but was not influenced by weight, height, BMI and time of the day. These observations allowed us to establish normal values of nNO, using a widely available handheld analyzer. This study may broaden the use of nNO measurement as a screening technique for PCD and improve case finding in adults with bronchiectasis.

NO is a gas produced by three isoenzymes of NO synthase (NOS) via the oxidation of the amino acid L-arginine to L-citrulline in the presence of nicotinamide adenine dinucleotide phosphate, oxygen and others cofactors (24). Two of these isoenzymes (NOS1 and NOS3) are called *constitutive NOS*. They are active only when the concentration of calcium increases. The last one (NOS2) is called *inducible NOS* and is calcium-independent. All 3 isoenzymes are expressed in the respiratory epithelium. In the respiratory system, NO is involved in regulation of blood flow, mucus secretion, ciliary motion, platelet function, immunity and neurotransmission (25,26).

A significant decrease of nNO has been observed in patients with PCD compared with HS (1–14). Several hypotheses have been proposed to explain the low nNO values in patients with PCD, including nNO "trapping" by viscous mucus, uptake by bacteria, or reduced expression or low activity of NOS isoenzymes (1,24,25). It has also been suggested that nNO is sequestrated in obstructed paranasal sinuses, or that its synthesis is reduced due to agenesis of paranasal sinuses (23,24,27). Hence, an important part of nNO comes from the paranasal sinuses. Baboons constitutively devoided of paranasal sinuses have extremely low nNO concentrations, while humans, rhesus monkeys and elephants, which possess paranasal sinuses, have much higher nNO concentrations. This suggests that paranasal sinuses are an anatomic requirement for nNO production (28). Aplasia or hypoplasia of frontal and/or sphenoidal sinuses is common in patients with PCD. One study showed that frontal and/or sphenoidal sinuses were either aplastic or hypoplastic on CT scans in 30 out of 41 (73%) patients with PCD (27). This could provide an explanation for low nNO in PCD, but requires further studies.

Although most patients with PCD have low nNO values, several studies have also highlighted that 3 to 7 % of patients with PCD have normal or even increased nNO concentrations (3,9,12,27). Indeed, one of our PCD patients has nNO values slightly above the lower limit of normal as defined in the present study by the 2.5 percentile. By providing normal values of nNO, our data will allow to study in more detail the sensitivity and specificity of nNO to detect patients with PCD.

The main finding of the present study is that age was significantly and negatively correlated with nNO. For each increase in age of 1 year, nNO_{adj} decreased of about 4 ppb. Two previous studies did not find any relationship between nNO and age (29,30). In the present study, gender, weight, height, BMI and time of the day were not correlated with nNO. Our findings are in agreement with previous studies,

which also did not find any association between gender and nNO (29–31). The effects of weight, height and BMI on the values of nNO have not been previously assessed. Regarding time of the day, a circadian effect on nNO was found in one study (32) but not in another one (30). No relationship was generally found in the present study between nNO and month of the year except for a reduction in May, when using January as an arbitrary reference. The significance of this finding is unclear at the present time. One can hypothesize that seasonal allergens may have influenced nNO measurements. However, NO being a mediator of inflammation, increased nNO values would have been expected in allergic rhinosinusitis. Furthermore, sinusitis was an exclusion criterion for this study. Also, the number of patients evaluated in May was small (n=9) and no attempts have been made to systematically stratify nNO measurements according to month of the year. Further studies are needed to clarify this issue.

One limitation of this study is that, despite a rigorous measurement protocol, the nNO sampling conditions were not strictly controlled. Indeed, there was no certainty as to the complete closure of the soft palate during sampling time. Also, only one flow rate was tested (5 ml/s), mainly because of its shorter sampling time (45 sec as compared to 2 min at a flow of 2 ml/s). In the present study, nNO values were moderately but significantly associated with ambient NO. To remove this source of bias, and as recommended in several articles, ambient NO was subtracted from the raw values of nNO (15,20,31,33) to obtain adjusted nNO values truly reflecting nasal NO production. Another limitation is that our study was not designed to control for circadian and seasonal variations of nNO, and the possible effects of these parameters were only assessed retrospectively. Although no major effect has been observed, the occurrence of small effects could not be ruled out.

Normal nNO values that we have determined for the NIOX MINO are certainly different from those measured with other devices, including those using chemiluminescence. Sampling of NO measured with the NIOX MINO differs significantly from conventional methods, as not the peak NO value, but the NO concentration of the total volume sampled is determined. In addition, the sampling period of a minimum of 45 seconds is much longer compared to conventional devices in which the maximum nNO level is usually reached after 10 to 20 seconds. In addition, only adults have been included in this study, and normal nNO values for children have not been determined. The reference values suggested in this article are therefore only valid for this particular analyzer and for adults. Also, the negative predictive value of a normal test is not precisely known, and should be studies in a large cohort of PCD. Despite these limitations, the NIOX MINO has the advantage of being portable and relatively inexpensive in

comparison to conventional stationary chemiluminescence analyzers. Additionally, it is already widely used in medical offices for the measurement of exhaled NO in patients with asthma. It can thus easily be adapted for measurement of nNO in these facilities. For these reasons, the NIOX MINO is an interesting screening tool for PCD. We believe that the present study can significantly broaden the use of nNO measurement to improve case finding in patients with suspicion of PCD, and thus prompt further diagnostic investigations. Further studies are needed to determine nNO values in a larger sample of adults with PCD, in children with PCD, as well as in patients with other causes of bronchiectasis.

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Table 1: Intraclass correlation coefficient and comfort score in 3 methods of nNO sampling (n=25).

	ICC		Comfort score	
	mean	95% CI	mean	SD
Breath hold (BH)	0.86	0.78-0.95	1.56	0.77
Tidal breathing through straw (TB-S)	0.94	0.91-0.98	2.28	0.79
Tidal breathing through continuous positive airway pressure apparatus (TB-CPAP)	0.90	0.84-0.96	2.16	0.75

ICC: intraclass correlation coefficient, CI: confidence interval, SD: standard deviation.

Table 2: nNO and ambient NO values in 200 adult HS expressed in parts per billion (ppb).

	N	mean	SD	median	Interquartile range
Raw nNO (average of 3 measurements)	200	531	228	518	300
ambient NO	200	14	10	12	14
nNO adjusted to ambient NO (average of 3 measurements)	200	517	226	498	296

Table 3: Normal values of nNO in adults according to age, expressed in ppb.

Age (yr)	q2.5	q5	q25	q50	q75	q95	q97.5
20	201	257	441	582	734	977	1064
21	198	254	438	578	730	973	1060
22	195	251	435	575	726	969	1055
23	193	248	432	571	723	965	1051
24	190	246	428	568	719	960	1046
25	187	243	425	564	715	956	1042
26	184	240	422	561	711	952	1038
27	182	237	419	557	708	948	1033
30	174	229	409	547	697	935	1020
31	171	226	406	544	693	931	1016
32	168	223	403	540	689	927	1012
33	166	220	400	537	686	923	1008
34	163	218	397	534	682	919	1003
35	160	215	394	530	678	915	999
36	158	212	391	527	675	911	995
37	155	209	388	524	671	907	991
38	152	207	385	520	667	902	986
39	150	204	381	517	664	898	982
40	147	201	378	514	660	894	978
41	144	198	375	510	656	890	974
42	142	196	372	507	653	886	969
43	139	193	369	504	649	882	965
44	137	190	366	500	646	878	961
45	134	188	363	497	642	874	957
46	131	185	360	494	638	870	953
47	129	182	357	490	635	866	948
48	126	179	354	487	631	862	944
49	124	177	351	484	628	858	940
50	121	174	348	481	624	854	936
51	118	171	345	477	621	850	932
52	116	169	342	474	617	846	928
53	113	166	339	471	614	842	924
54	111	163	336	467	610	838	919
55	108	161	333	464	607	834	915
56	106	158	330	461	603	830	911
57	103	155	327	458	600	826	907
58	100	153	324	455	596	822	903
59	98	150	321	451	593	818	899
60	95	147	318	448	589	815	895
61	93	145	315	445	586	811	891
62	90	142	312	442	582	807	887
63	88	140	309	439	579	803	883
64	85	137	306	435	575	799	879
65	83	134	303	432	572	795	875
67	78	129	297	426	565	787	867
69	73	124	292	419	558	780	859
70	70	121	289	416	555	776	855
71	68	119	286	413	551	772	851
75	58	109	274	401	538	757	835
76	55	106	271	398	534	753	831
77	53	103	269	394	531	749	827
80	46	96	260	385	521	738	815

Figure 1: Normal values of nNO according to age.

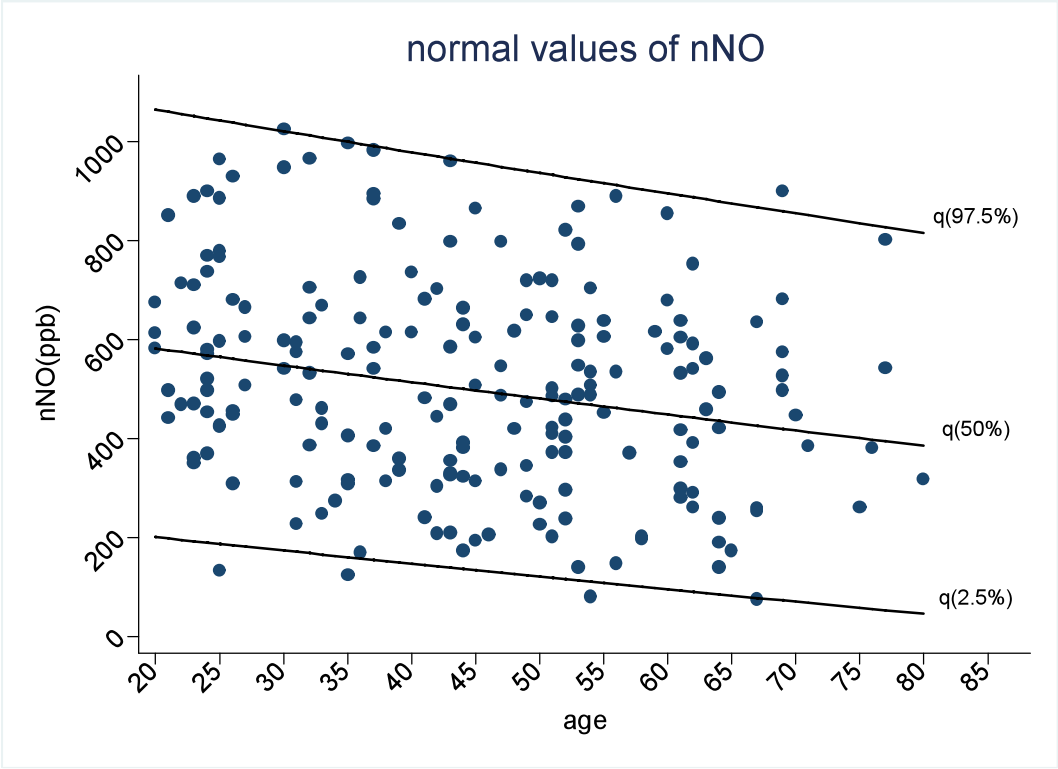
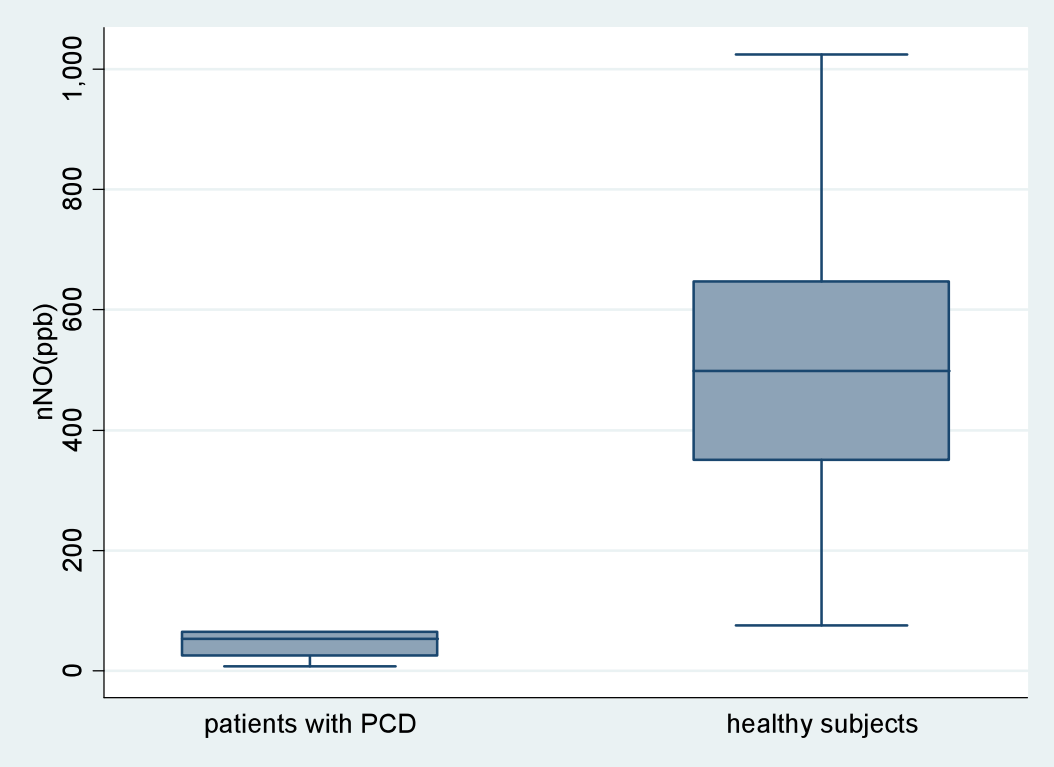


Figure 2: nNO values in adult HS (n=200) and patients with PCD (n=7).



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