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Abstract

Nasal Nitric Oxide (nNO) measurement is recommended to screen for Primary Ciliary Dyskinesia (PCD) in subjects with suggestive history and symptoms. Clinical use of alternative methods (*ie* breath hold (BH), tidal breathing (TB)) in children unable to perform the gold standard slow Exhalation against a Resistance (ER) method has not been sufficiently evaluated.

We extracted retrospectively (2013-2019) 454 files (374 subjects) containing nNO results. Median [IQR] age at inclusion was 7.0 [4.7 – 11.0] years, 105 (28.1%) children were younger than 5 years. ER or BH methods were more frequently mastered by children older than 5 years compared to younger children (69.4% and 52.7% versus 21% and 5.6%, respectively, $P < 0.0001$), the latter succeeding only in TB measurement in 77.4% of cases. In 130 files with both ER and BH measurements (nNO-ER and nNO-BH), nNO-BH was 102 [96.2;108.3]% that of nNO-ER. In 175 files including nNO-ER and nNO-TB measurements, nNO-TB was 64.4 [IQR: 53.7;80.4]% that of nNO-ER with an excellent correlation between nNO values (r = 0.94 [95%CI 0.91;0.95]; $P<0.0001$) and discordance in the interpretation of nNO results in 16 (10.2%) cases.

Final PCD diagnosis was similar in patients included before or after 5 years of age (confirmed 16 (15.2%) and 48 (17.8%); excluded 81 (77.1%) and 192 (71.4%), respectively; $P = 0.32$).

In conclusion, reliable nNO-BH and nNO-ER results are interchangeable. Children tested with ER or with TB method have similar final PCD diagnosis. Alternative methods to measure nNO might be studied further for use in clinical practice.

Introduction

Primary Ciliary Dyskinesia (PCD) is a congenital disease that causes early onset of respiratory symptoms, contrasting with a usually delayed diagnosis. In the absence of a simple and unique gold standard test for diagnosing PCD in patients with suggestive clinical symptoms, European and North American working groups recommend first to exclude any differential diagnosis, then to perform nasal Nitric Oxide (nNO) measurement along with other tests such as high-speed video microscopy, ciliary ultrastructure or genetic analyses [1-3].

It is now well established that patients with Cystic Fibrosis or PCD have very low nNO output, though the reason for this remains elusive, especially since few PCD patients with specific mutations have a normal level of nNO [4]. However, the measurement of nNO remains the easiest technique to screen the majority of PCD patients.

In order to decrease the age at diagnosis, it is necessary to gather evidence on the feasibility, reliability and clinical utility of nNO obtained in younger children, especially when the gold standard method (slow exhalation against a resistance (ER)) for nNO measurement is difficult to obtain or to interpret.

False positive nNO results (low nNO value in children without PCD), the most problematic outcome, are frequently ascribed to obstructive rhinitis, which should be detected by examining the child's nose before measurement [5]. They can also be suspected in the presence of irregular NO traces, therefore it is necessary to repeatedly check any low nNO value [5]. Another cause for false positive nNO tests in young children could be the use of an inadequate (too high) threshold to discriminate PCD from non-PCD children. Airway NO production is maximal in paranasal sinuses [6] and increases along with paranasal sinuses

development during the first decade, reaching adult values thereafter [7]. As a consequence, thresholds of nNO established in subjects over 12 years of age may not be suitable for young children with physiologically lower NO output [8]. Moreover, thresholds established for methods involving velum closure (ER or breath hold (BH)) are higher than those computed using the tidal breathing (TB) method, the latter often being the only method available in young children [9-11]. Finally, alternatives to the gold standard ER method (*ie* BH and TB methods) have been used in children, with excellent sensitivity and specificity [13-18]. However, the proportion of children able to master BH or TB but not ER method, along with the clinical usefulness of these alternative methods, have not been evaluated in routine practice.

In order to describe the use in routine practice and the potential clinical impact of nNO measured using BH or TB methods (nNO-BH and nNO-TB, respectively) in young children, we set up a retrospective study on a large population screened for PCD.

Subjects and methods

We retrieved retrospectively (2013 to 2019) all files containing nNO results from the database of a pulmonary function test department located in a tertiary hospital. Since that hospital is a national reference center for PCD diagnosis, children are sent from all over the country for a work-up. Families were informed of the possible retrospective use of their children's results (declared to the French authority for data protection, CNIL) and gave oral consent. The database contains all routine nNO tests results deemed technically reliable and could include one, two or three results per file, according to the methods used

(ER and/or BH and/or TB). We recorded other tests performed as part of the patients' diagnosis work-up and we noted the final diagnosis.

Material

NO was measured online using a chemiluminescence NO analyzer (NIOX Flex, Aerocrine Solna, Sweden, in 2013 and 2014, then CLD 88sp NO-analyzer, Eco Medics AG, Duernten, Switzerland from 2015 to 2019, with a flow rate sample of 0.3 $L.min^{-1}$ and 0.33 $L.min^{-1}$, respectively). Real-time NO curve was displayed on the screen. Ambient NO was recorded before each test.

Exhaled NO measurement

Before nNO measurement, children over 3 years tried to perform an online measurement of exhaled NO (eNO) at a 50 $mL.s^{-1}$ expiratory flow (bronchial eNO) in order to assess their chance of success with the ER method, as that method requires a similar respiratory maneuver (Figure E1).

Nasal NO measurements

Transnasal nNO measurements were performed as previously described [18] in a seated child (sometimes in a lying sleeping infant) with an olive inserted in one nostril entraining air from the other nostril. Subjects were constantly exhaling (through the machine circuit or through a party blower) against a resistance of 8 to 10 cmH_2O (ER method) or breath holding (BH method) at Total Lung Capacity during a Valsalva maneuver to achieve velum closure until an NO plateau was reached (maximum - minimum NO values within 10% of the mean). nNO value was the mean of an at least 3 s duration plateau. During tidal breathing (TB method), subjects breathed regularly, with mouth closed for most of them, which determined a succession of variations in the NO concentration on the trace.

Measurements during crying were not reported whereas measurements during sleep were recorded when the breathing pattern was regular (peaks within 10%). The mean of 5 peaks during regular breathing was recorded. Measurements were performed in both nostrils and the mean value was reported, except when the difference between the two nostrils exceeded 10% and then the highest value was recorded.

It is to be noted that some early files (2013-2104) were part of a previous prospective study [18] during which we performed TB method in all patients. After this study, we set up a Standard Of Procedure (SOP) according to which children less than 8 years of age always performed a nNO-TB measurement first while older patients were first tested with nNO-ER or nNO-BH methods (Figure E1). (See online Supplemental Material)

When nNO measure was low or performed with a high ambient NO, we proposed to control the measure, but a second visit often could not be arranged for children who lived far. Moreover, our dataset did not include visits that did not yield results (eg non-cooperative children or obvious and total nasal obstruction).

Final diagnosis

The final diagnosis was established according to the ERS recommendations [1], or after discussing the case with a multidisciplinary panel of experts attended by a clinician, a beat ciliary microscopist, a TEM microscopist, a physiologist, and a geneticist [19]. Among the 63 PCD children, 40 children had typical cilia ultrastructure defects, of which 35 also had bi-allelic pathogenic mutations in PCD genes (4 genetics results still pending), and 19 had a pathogenic genotype without abnormal cilia ultrastructure. In addition, three children with Kartagener Syndrome and low nNO (one with immotile cilia), and one child with

typical clinical presentation, low nNO and immotile cilia were considered as PCD by the panel.

Statistical analysis

The nNO thresholds used throughout the study period to take medical decision were those previously established for children 4 years and older (*ie* 82 nL.min^{-1} for nNO-ER and nNO-BH methods, and 40 nL.min^{-1} for nNO-TB) [18]. In children younger than 4 years (73 results in 61 children), the threshold used for nNO-TB was the lower limit of normal published by Adams and colleagues from birth up to 1 year (9 files in 8 infants) [10], progressively increased between 1 year and 4 years to smoothly fill the gap between 34 nL.min^{-1} and 40 nL.min^{-1} (Table E1).

Results were described as number (percentage), or median [Q1;Q3] (range). Proportions were compared using the Chi-2 test. Comparisons between paired and unpaired data were performed using the Wilcoxon matched-pairs rank test or the Mann-Whithney test, as appropriate. Correlation between nNO-ER and nNO-TB was established using the Spearman's test. We performed Bland and Altman plots to investigate the inter-method agreement. Receiver Operating Characteristic (ROC) curves were constructed to look for the best nNO threshold (Youden test) in specific age groups. Statistics were performed using GraphPad Prism (version 6.01). *P* value < 0.05 was considered as significant.

Results

We retrieved 454 files belonging to 374 children or young adults with a median [Q1;Q3] (range) age of 7.0 [4.7;11.0] (0.3 – 19.4) years at inclusion, including 105 (28.1%) children

aged less than 5 years at the time of measurement (Figure 1). The files were evenly distributed across years, except for 2014 when we changed NO analyzer (Figure E2).

Among the 412 tests performed in 336 patients 3 years or older, 262 (63.6%) tests included a bronchial eNO result. Children who succeeded at this measurement were significantly older (9.9 [7.0; 12.9] versus 7.4 [4.2; 6.7] years; $P < 0.0001$) and succeeded more frequently at ER method than children who failed bronchial eNO manoeuver (208 (79.4%) versus 44 (29.3%); $P < 0.0001$). (see Online Supplementary Material)

The dataset included 255 nNO-ER, 181 nNO-BH and 361 nNO-TB measurements (Figure 2). There was more than one visit in 57 (15.2%) children, of whom 41 (71.9%) had two visits during which they performed one, two or three methods each time (26, 13 and 44 duplicate results for ER, BH and TB methods, respectively (see Online Supplementary Material). The proportion of patients with repeated measures was similar between those under and over 5 years at the time of inclusion (20% versus 13.4%, $P = 0.15$). (see Online Supplementary Material)

The number of measurements according to age and methods performed is given in Table 1. As a consequence of the SOP we used (Figure E1), not all between-group comparisons were relevant. There was a significantly larger proportion of patients over 5 years able to master ER (69.4%) or BH (52.7%) method compared to children younger than 5 years (21.0% and 5.6%, respectively; $P < 0.0001$ for both methods), and a larger proportion of patients aged less than 5 years ending up with only a nNO-TB result compared to older patients (77.4% versus 15.7%, $P < 0.0001$). The age distribution of patients able to perform the BH method but not ER, or only the TB method is shown in Figure 3.

At inclusion, nNO-TB, nNO-BH and nNO-ER were measured higher than the discriminant threshold in 202/295 (68.5%), 101/150 (67.3%) and 140/207 (67.3%) patients, respectively (P = 0.95). In 130 files including both nNO-ER and nNO-BH results, the value of nNO-BH was 102 [IQR: 96.2;108.3]% that of nNO-ER without significant difference between the two nNO values (P = 0.08) (Figure 4a). In four (3.1%) of these files, the interpretation of the result according to the threshold was discordant between nNO-ER and nNO-BH. In 175 tests that yielded nNO-ER and nNO-TB results, the value of nNO-TB was 64.4 [IQR: 53.7;80.4]% that of nNO-ER with an excellent correlation between nNO values (r = 0.94 [95%CI 0.91;0.95]; P < 0.0001) (Figure 4b). In 19 tests (10.9%) the nNO-TB value was higher than the nNO-ER value, in favor of an unreliable nNO-ER measurement or of an undetected significant nasal obstruction. Apart from these 19 unreliable tests including two discordant results, there were 16 (10.2%) cases of discordance in the interpretation of nNO-ER and nNO-TB.

Between patients younger and older than 5 years at inclusion, the diagnosis of PCD was equally confirmed (15 (14.3%) versus 48 (17.8%), respectively; P = 0.50) or excluded (82 (78.1%) versus 192 (71.4%), respectively; P = 0.23) (Table 2). Ciliary beating analysis and genetics studies were performed in similar proportions in these two groups of patients (58 (55.2%) versus 174 (64.7%) for cilia beats, P = 0.12, and 19 (18.3%) versus 63 (23.5%) for genetics studies, P = 0.34, respectively) whereas respiratory biopsies for ciliary ultrastructure analysis were more frequently performed in patients over 5 years (P = 0.0002) (Table 2). Results on final work-up according to nNO methods successfully performed in children younger than 5 years of age are given in Table E3.

ROC analyses were performed using a maximum of one result per method for each patient. In patients 5 years or older, sensitivity and specificity of nNO measurement to discriminate PCD disease were 84.6 (95%CI 73.3; 95.9)% and 92.4 (95%CI 87.9; 96.9)%, respectively, for nNO-ER (n=171), and 86.1 (95%CI 74.8; 97.4)% and 87.9 (95%CI 81.4; 94.3)%,), respectively, for nNO-BH (n=135). In children under 5 years (n=90), nNO-TB sensitivity and specificity were 76.9 (95%CI 54.0; 99.8)% and 85.7 (95%CI 77.9; 93.5)%, respectively. The low sensitivity of the TB method was explained by three false negative tests (values higher than the threshold in PCD children) in two children with RSPH1 or CCDC103 mutation, and in a child with Kartagener syndrome and pending genetic results (see Online Supplementary Material). In 230 children (47/230 PCD) younger than 12 years, the ROC analysis of nNO-TB results did not show a different threshold from what we previously established[18] and used in this study (Table E2, see Online Supplementary Material).

Median [Q1;Q3](range) of ambient NO (NOamb) measured before 449 (99%) tests was 6.4 [1.9;19](0-126) ppb (Figure E2). Correcting for NOamb by subtracting it to nNO measures would have changed the interpretation of nNO in 16 (3.6%) tests performed in 15 children (Table E3). (see Online Supplementary Material)

Discussion

In this retrospective study including 454 nNO measurements performed in 374 children and young adults suspected of PCD, the TB method was the only method feasible in 77.4% of children younger than 5 years, of whom 21% could perform the gold standard ER method. Conversely, the BH method succeeded in only 14.8% of the patients 5 years or

older unable to perform ER method, but the results of these two methods proved interchangeable. Frequencies of corroborative tests undertaken and final diagnoses were similar between the patients included before and after 5 years, except for TEM, which was more frequently undertaken in older patients.

Because nNO measurement is a simple, highly sensitive and specific noninvasive test to screen children for PCD, it is recommended by the American Thoracic Society (ATS) and by the European Respiratory Society (ERS) [1,3]. However, measuring nNO early in life cannot always rely on the gold standard ER method because the younger the child is, the more frequently he or she would fail at this method (before 5 years for ATS, and before 6 years for ERS). In this context, the feasibility and clinical usefulness of alternative methods to assess nNO in routine practice should be considered. Adding to the difficulty of interpreting nNO values, nNO production in healthy children increases from birth up to 12 years of age (along with the development of nasal sinus) before leveling off [6,7,10,11,20]. nNO-TB increases quickly from very low values in the first month of life (17.7 [95%CI: 8.8;35.6] [10] and 15 [IQR: 9.6;22.8] [11] $nL.min^{-1}$) to four time this value at 1 year (56.4 [IQR: 36.3;75.2] [10] and 69.4 [95%CI: 34.0;142.0] [11] $nL.min^{-1}$); it then less than doubles during the second year of life (95.4 [IQR: 67.0;128.4] $nL.min^{-1}$ at 2 years [10]). These low initial values with large inter-individual variability might be impacted by any change in nasal flow or in NOamb, complicating the discrimination of PCD in infancy, as we found.

The BH method has been used in children and in adults with a 100% feasibility [13-17,21-24], but it is less adapted in young children: only 2 (3.2%) out of 62 preschoolers (4.1 to 6 years) and 43 (14.7%) out of 293 healthy children (3 to 7.2 years) were able to complete it [8,25] (see Online Supplemental Material). Therefore, the advantage of BH method would be

to offer an alternative method for school-aged or older patients unable to perform nNO-ER. nNO-BH has frequently been found higher or close to nNO-ER in studies which compared both measures in healthy or sick subjects (*eg* in healthy adults or children nNO-BH versus nNO-ER were, respectively, 201.3 versus 228.9 $nL.min^{-1}$ (P=0.1)[24]; 272.4 versus 236.4 $nL.min^{-1}$ (mean difference 3.5 ± 53.5) [8]; 366.5 versus 393.7 $nL.min^{-1}$ [17]; and 90.1 versus 8.9 $nL.min^{-1}$ [23]). To ensure velum closure during BH, it is proposed to concomitantly measure nasal CO_2 [12], but this technique is not available in all settings. Theoretically, there should be no difference between nNO values obtained with any method involving velum closure and no superimposed nasal airflow (no humming), while in case of communication between the nose and the pharynx a lack of plateau achievement or a plateau with low level of nNO should be seen. Any nNO-BH result that is low or around the threshold could be confirmed by a nNO-TB measurement (Figure E1), whose result is expected to be around half to two thirds that of nNO-BH [26]. By contrast, high above the threshold nNO-BH results without technical issue (low ambient NO) could be considered as reliable as nNO-ER results and of clinical usefulness.

Regarding the TB method, we confirmed: i) the high success rate in children younger than 5 years (95.2% [8], present study 97.6%), while a minority of these children could achieve nNO-ER measurement; ii) the excellent correlation between nNO-TB and nNO-ER results obtained in same patients; iii) the good concordance with ER method in terms of interpretation of the results. Our point was to assess whether nNO measurements performed before the age of 5 (mostly using the TB method) would result in a similar work-up for PCD diagnosis, which it did, except for TEM. We probably performed respiratory biopsy less frequently in the youngest because of the difficulty to obtain a correct sample before

10 years of age [27]. Respiratory biopsy is an invasive procedure which cannot be repeated too often, especially in young children for whom we may have reasoned differently (more observation times, genetic analysis performed earlier).

Sensitivity and specificity were similar across the three methods used to measure nNO, but slightly lower than that reported in a meta-analysis of 13 prospective studies (sensitivity 0.95 (95% CI 0.91–0.97), specificity 0.94 (95% CI 0.88–0.97)) [28]. As previously explained, we could not check all low nNO results: some of them were probably false positive results due to mild nasal obstruction, while some false negative TB tests could be ascribed to specific mutations in PCD children, or to high ambient NO in some cases.

The issue of high level of ambient NO during nNO measurements has received little attention so far. In healthy children, it has been evaluated that ambient NO accounts for half of its value in nNO-BH result [7]. In another multicenter study, the impact of ambient NO was found to be non-significant in the town with persistently low ambient NO level (5.0 [95%CI 14.0 to 28.4]%; $P = 0.63$). In opposite, in the two towns where ambient NO could reach 100 ppb, ambient NO significantly impacted nNO, but with a large variability, (24.2 [95%CI 4.9 to 47.0]%; $P = 0.015$, and 19.8 [95%CI 7.7 to 33.3]%; $P = 0.001$) [29]. Recommendations are to provide NO-free air for nNO measurement, or to record the ambient NO for each test in order to take it into account [12]. But no study has evaluated settings aimed at providing NO-free air during nNO measurement in children, and there are no consensual guidelines on how to take ambient NO into account in children. In adult subjects, it is proposed to subtract 100% of ambient NO to the measurement [30]. From our results, we can state that ER and BH methods are less impacted by ambient NO than TB method. Therefore, ambient NO will influence nNO interpretation in the youngest with

physiological low nNO level and often only nNO-TB result. It can also influence the interpretation of nNO in all subjects with borderline values measured in settings where ambient NO is high.

Our study has several limitations. First, we did not intend to rate the real feasibility of different methods to measure nNO in children, which would have required the inclusion of all attempts of nNO measurement without result available. The feasibility of different methods has already been published [8,13-17,21-25], and we focused on which methods were successful according to the patient's age and on the relationships between methods used and work-up or final diagnosis. Second, the thresholds we routinely used during the study were higher than those proposed by international guidelines (*eg* 82 versus 77 $nL.min^{-1}$ for ER method), which were not available at the beginning of the study. Instead, throughout the study we used the thresholds previously validated in our center [18]. As our aim was to retrospectively assess the clinical usefulness of nNO results interpreted as we did at the time of measurement, it would not have been relevant to show our results according to different thresholds. Lastly, we were not able to assess to what extent the work-up had truly been influenced by nNO result because we could not track the exact order of the tests. However, the proportion of children with nNO results higher or lower than the discriminant threshold was similar across the three methods evaluated, as well as among children with PCD diagnosis confirmed or excluded, independently of the patient's age. Moreover, there were no differences in PCD diagnosis or in frequencies of corroborative tests performed according to patients' age (except for TEM) (Table 2) or nNO methods used (Table E3).

Our results are in favor of mastering all methods available to measure nNO in children. It is probable that the youngest will not be able to perform the gold standard nNO-ER, whose

threshold might not be adapted to their age, and that some older children will fail the ER method. The inability to perform a bronchial eNO measurement could help indicate which patients will require alternative methods for nNO measurement. In this case, a correct nNO-BH measurement, especially when corroborated by the nNO-TB result, could be considered as usable in clinical practice. In settings without resources for ciliary beat analysis, nNO measurement might be of utmost importance to guide the work-up, especially in the youngest in whom respiratory biopsies are less frequently successful. Standardization of alternative methods to measure nNO along with the procedure to take into account ambient NO are currently lacking. The present evaluation of the clinical usefulness of these methods in routine practice should raise interest in developing such guidelines.

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TABLES

Table 1 – Comparisons between proportions of nasal Nitric Oxide measured using different methods in 124 children under 5 years and in 330 children 5 years or older

Results are numbers *(%)**	Tests performed < 5 years	Tests performed ≥ 5 years	*P*-value
Number of visits	124	330	
nNO-ER measures	26 *(21.0)*	229 *(69.4)*	<0.0001
nNO-BH measures	7 *(5.6)*	174 *(52.7)*	<0.0001
Visits with nNO-BH and no nNO-ER	2 *(1.6)*	49 *(14.8)*	<0.0001
nNO-TB measures	121 *(97.6)*	240 *(72.7)*	<0.0001
Visits with only nNO-TB	96 *(77.4)*	52 *(15.7)*	<0.0001

nNO: nasal Nitric Oxide; nNO-ER: NO measurement performed during an expiration against a resistance: nNO-BH: NO measurement performed during a breath holding; nNO-TB: NO measurement performed during tidal breathing

*: percentages of visits in the age group

Table 2 – Frequency of work-up tests and final diagnosis in 374 study children according to age at inclusion

Results are numbers *(%)*[*]	Children < 5 years at inclusion	Children ≥ 5 years at inclusion
Children included	105	269
Work-up		
Nasal brushing	58 *(55.2)*	174 *(64.7)*
TEM	34 *(32.4)*	147 *(54.6)*[†]
Genetics studies	19/104 *(18.3)*	63/268 *(23.5)*
Final diagnosis		
PCD confirmed	15 *(14.3)*	48 *(17.8)*
PCD excluded	82 *(78.1)*	192 *(71.4)*
Pending cases	6 *(5.7)*	25 *(9.3)*
Unconcluded cases	2 *(1.9)*	4 *(1.5)*

TEM: transmission electron microscopy on nasal or bronchial biopsy

[*]: percentages are related to the population defined in the top cell of each column except for genetic studies outcome where missing data were present (the total number of patients with a known outcome is the denominator)

[†]: Compared to children younger than 5 years of age; $P = 0.0002$

Figures

Figure 1 – Age distribution at inclusion in 374 children and for all measurements recorded in the study (454 tests)

Number of children at inclusion (black columns) and at all measurements recorded (grey columns)

Figure 2 – Distribution of 454 tests including one, two or three results of nasal Nitric Oxide according to the methods of measurement used

nNO: nasal Nitric Oxide; nNO-ER: NO measurement performed during expiration against a resistance: nNO-BH: NO measurement performed during breath holding; nNO-TB: NO measurement performed during tidal breathing

Figure 3 – Age distribution according to methods of nasal Nitric Oxide measurement succeeded by the patients

Tests included: only nasal Nitric Oxide measures using the Tidal Breathing method (black columns); Breath Hold result but no Exhalation against a Resistance result (striped columns); Exhalation against a Resistance result whichever other methods successfully performed or not on the same occasion by the patient (grey columns)

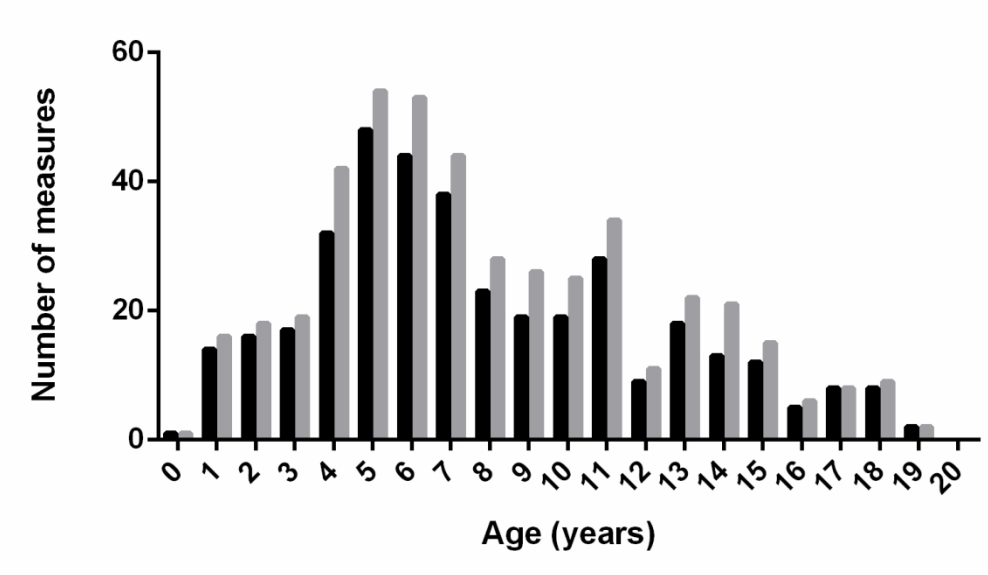
Figure 4 – Bland Altman graphs comparing nasal Nitric Oxide measured using the Expiration against a resistance and alternative methods

Figure 4a – Bland Altman graph comparing the Exhalation against a Resistance with the Breath Hold method in 130 patients

nNO: nasal Nitric Oxide; ER: Exhalation against a Resistance; BH: Breath Hold

Figure 4b – Bland Altman graph comparing the Exhalation against a Resistance to the Tidal Breathing method in 175 patients

nNO: nasal Nitric Oxide; ER: Exhalation against a Resistance; TB: Tidal Breathing



age distribution of tests

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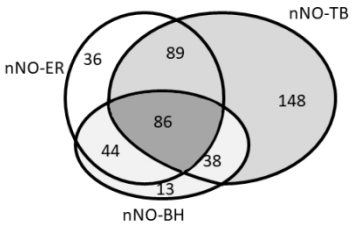

Venn diagram of included nasal No measurements

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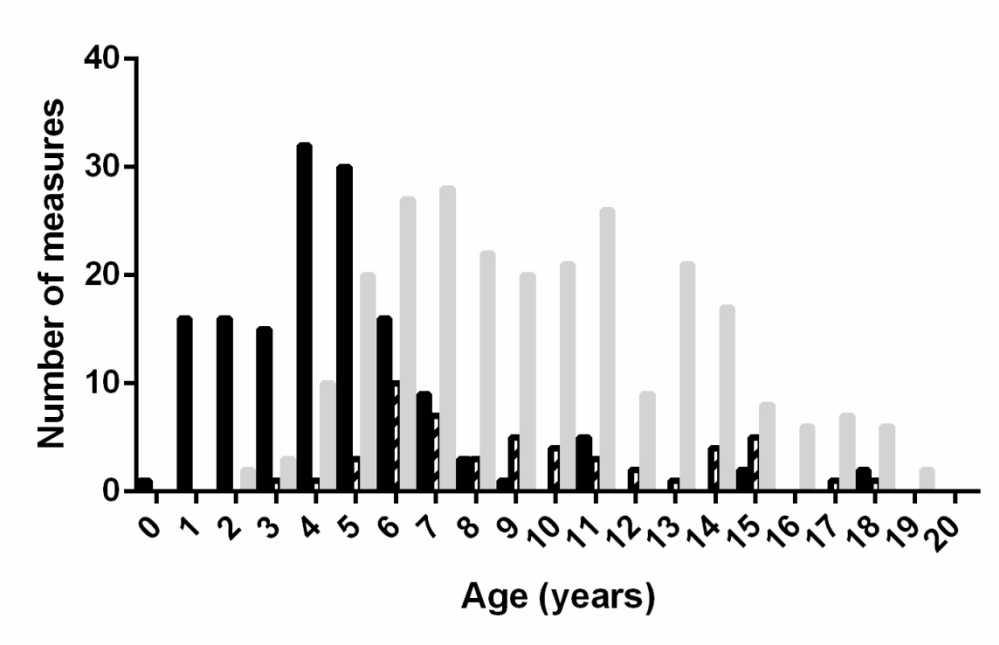

Distribution of age according to methods used to measure nasal NO

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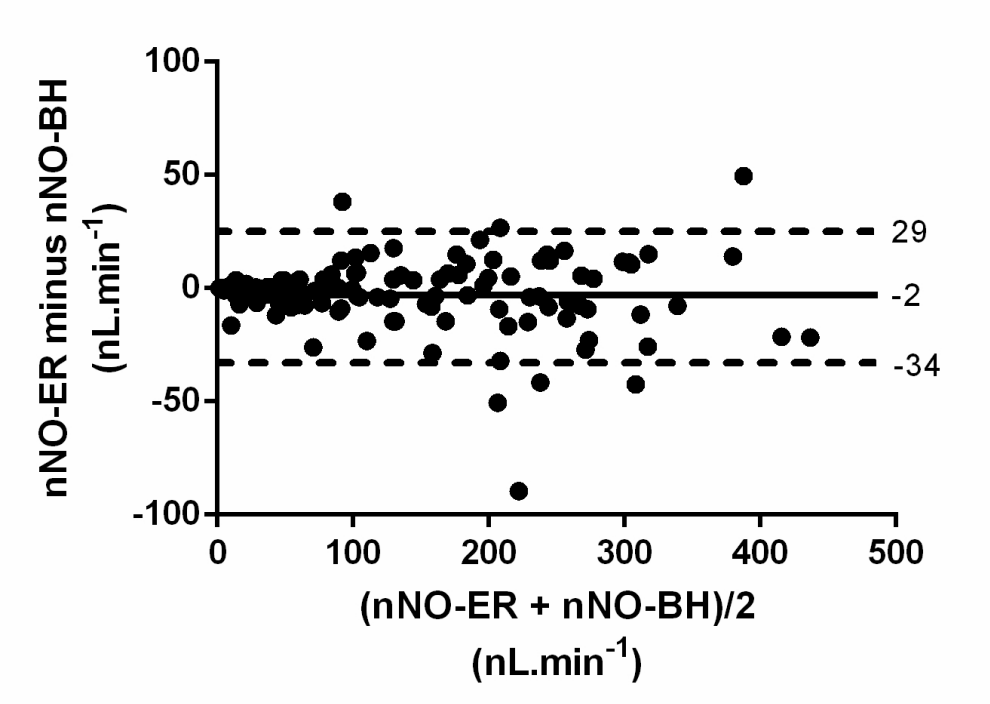

Bland Altman nNO-ER and nNO-BH

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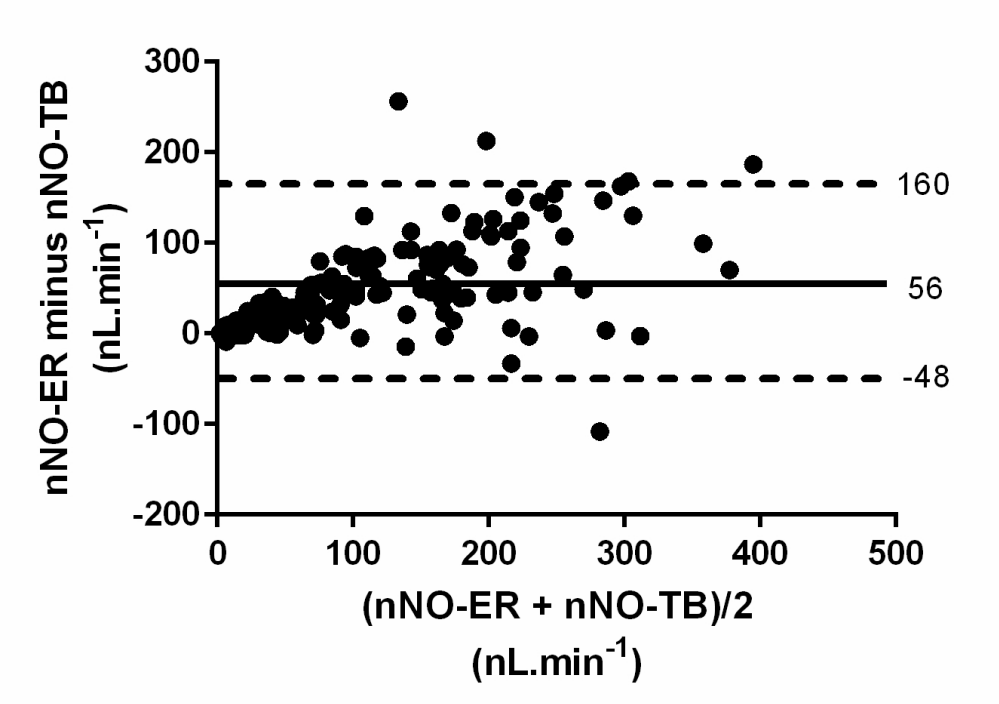


Bland Altman for nNO-ER and nNO-TB

112x79mm (300 x 300 DPI)

Breath holding and tidal breathing nasal NO to screen children for Primary Ciliary Dyskinesia

Nicole Beydon, Aline Tamalet, Estelle Escudier, Marie Legendre, Guillaume Thouvenin

Online Supplementary Material

Methods

According to our a Standard Of Procedure (SOP) children under 8 years of age always performed a nNO-TB measurement first while older patients were first tested with nNO-ER or nNO-BH methods (Figure E1). In case of failure to achieve ER and BH methods or in case of low/borderline results, nNO-TB was measured (in order to confirm low level or to reach for evidence of a blocked nose when nNO-TB was higher than nNO-ER or nNO-BH).

Results

Among the 413 tests performed in patients 3 years or older, 262 (63.4%) tests included bronchial eNO result. nNO-ER result was available in 208 (79.4%) cases with successful eNO measurement, whereas it was available in only 44 (29.3%) cases among the 150 tests without eNO result ($P < 0.0001$). The likelihood ratio for a patient to succeed at nNO-ER measure when eNO was possible was 2.4. As expected, patients without eNO were significantly younger than those able to perform a correct eNO measurement (7.4 [4.2;6.7] versus 9.9 [7.0;12.9] years; $P < 0.0001$). It is to be noted that eNO values measured in PCD patients were lower than those obtained in patients in whom PCD was excluded (median [Q1;Q3] 4.1 [2.7;6.4] versus 8.0 [5.9;13.7]; $P < 0.0001$).

The dataset encompassed repeated nNO measurements in 57 (15.2%) children, consisting in most of the cases in 2 measurements per child (41 cases, 71.9%). Measures of nNO were repeated at least twice using the same method in 26 (12.6%), 13 (8.7%) and 44 (14.9%) children for ER, BH or TB methods, respectively. The medians [Q1;Q3] delay between the two measurements were 12 [6 ; 18.2], 11 [2.5 ; 21], and 12.5 [5.2 ; 21.5] months for ER, BH and TB methods, respectively, and medians [Q1;Q3] of differences (second minus first measurement) were 11.3 [-0.6 ; 77.9], 8.6 [-5.3 ; 62.0], and 10.0 [-2.3 ; 31.6] $nL.min^{-1}$, respectively (Figures E4). The proportion of children with repeated measures was similar between children first tested under 5 years or patients seen at first at 5 years or older (20% versus 13.4%, $P = 0.15$) (Table 1). Among the 21 children seen at first before 5 years and with repeated measures, 9 children had at least one measure after 5 years of which 5 had only one measure before 5 years of age.

Sensitivities and specificities were calculated for the three methods, using a maximum of one result per method for each patient. In case of more than one result per method in a child, we used the highest value obtained with ambient NO ≤ 20 ppb.

Low sensitivity of TB method was explained by three false negative tests (values higher than the threshold in PCD children) because of a RSPH1 or CCDC103 mutation in two children, or a child with Kartagener syndrome and pending genetic results. Age at measurement, nNO-TB and ambient NO values in these three children were, respectively: 3.5 years, 164 ppb (54 $nL.min^{-1}$) and 8.7 ppb; 3.9 years, 211 ppb (70 $nL.min^{-1}$) and 0 ppb; and 4.9 years, 319 ppb (96 $nL.min^{-1}$) and 1.9 ppb.

In 230 children (47/230 PCD) younger than 12 years or in 190 children (37/190 PCD) younger than 8 years ROC analyses did not show different thresholds for TB method

compared to the threshold we previously established [E1] and used in this study (Table E2). However, specificity appeared slightly better compared to that of children younger than 5 years.

In children less than 5 years of age with PCD diagnosis excluded, nNO-TB was equal to or higher than 40 $nL.min^{-1}$ in 72/86 (83.7%) cases, of whom 40 were less than 4 years. In the remaining 11 measurements performed under 4 years of age in children without PCD, nNO-TB was higher than the threshold calculated according to age (Table E1) in only one measurement.

Median [Q1;Q3](range) of ambient NO (NOamb) measured before 449 (99%) tests was 6.4 [1.9;19](0-126) ppb and superior to 20 ppb in 103 cases (22.9%) (Figure E3). The effect of NOamb was assessed by noting whether after subtracting it to the nNO measure, nNO value remained the same side of the threshold or not. Correcting NO for NOamb would have changed the interpretation of nNO in 16 tests performed in 15 children of which 12 were 9 years or younger at the time of measurement (Table E4). In three ER measurements (1.2% of ER measurements) and in three other cases of BH measurements (1.7% of BH measurements) nNO would decrease from above to under the discriminant threshold; whereas it would happen in 12 cases for TB measurements (3.3% of TB measurements, two cases with concomitant decrease of nNO-ER under the threshold, and one case with concomitant decrease of nNO-BH under the threshold). In seven cases (43.7%), ambient NO was 20 ppb or less, which means that the nNO measurements were close to the threshold. In five cases where nNO-VT had been evaluated on another occasion, three children showed a subsequent nNO-VT value above the threshold and two lower than the threshold.

Discussion

Corbelli and colleagues reported that among 58 children able to perform nNO-BH, four (6.9%) were unable to correctly achieve eNO measurement [E2] which requires the same respiratory maneuver than the ER measurement. This is close to the 14.8% of our study children who could perform nNO-BH but not nNO-ER, representing a small but true population for which nNO-BH was useful. Therefore, the advantage of BH method would not be to allow a decrease in the age of children performing nNO measurement but to offer an alternative method for school aged or older patients unable to perform nNO-ER.

References

E1. Beydon N, Chambellan A, Alberti C, de Blic J, Clément A, Escudier E, Le Bourgeois M. Technical and practical issues for tidal breathing measurements of nasal nitric oxide in children. *Pediatr Pulmonol.* 2015;50:1374–1382.

E2. Corbelli R, Bringolf-Isler B, Amacher A, Sasse B, Spycher M, Hammer J. Nasal nitric oxide measurements to screen children for primary ciliary dyskinesia. *Chest.* 2004;126:1054–1059.

E Tables

Tables E1 – Thresholds of nasal Nitric Oxide used for the tidal breathing method in children under 4 years of age

Age (years)	**Threshold ($nL.min^{-1}$)**
0.1	**9**
0.2	**11**
0.3	**13**
0.3	**16**
0.4	**18**
0.5	**21**
0.6	**23**
0.7	**26**
0.8	**29**
0.8	**31**
0.9	**33**
1.0	**34**
1.0 to 1.5	**34.5**
1.5 to 2.0	**35**
2.0 to 2.5	**36**
2.5 to 3.0	**37**
3.0 to 3.5	**38**
3.5 to 4	**39**
≥ 4	**40**

The Tidal Breathing value was the mean of 5 peaks during regular breathing. Thresholds are the Lower limit of normal from birth to 1 year of age, the previously published threshold for children 4 years or older [E1] and a smooth connection between 1 and 4 years of age.

Table E2 – Thresholds of nasal Nitric Oxide measured using tidal breathing method in 230 children younger than 12 years and in 190 children younger than 8 years

	PCD confirmed	PCD excluded	AUC (95%CI)	Sensitivity (%)	Specificity (%)	Threshold ($nL.min^{-1}$)
Children < 12 years	47	183	0.88 (0.82;0.94)	76.6	89.1	40.8
Children < 8 years	37	153	0.88 (0.80;0.95)	78.4	90.9	40.8

PCD: Primary Ciliary Dyskinesia; AUC: Area Under the Curve; CI: Confidence Interval

Table E3 – Frequency of work-up tests, final diagnosis and Tidal Breathing nasal Nitric Oxide results in children younger than 5 years at inclusion according to the method of nasal Nitric Oxide measurement they succeeded

Results are numbers *(%)*[*] nNO-TB median [Q1;Q3] (ppb) [#]	Children < 5 years with nNO-ER	Children < 5 years with only nNO-TB
Children included nNO-TB	24 n = 23,174 [49;281]	79 214 [88;340]
Work-up		
Nasal brushing nNO-TB	12 *(50.0)* 145 [33;220]	44 *(55.7)* 155 [57;306]
TEM nNO-TB	8 *(33.3)* 56 [33;199]	26 *(32.9)* 76 [42;198]
Genetics study nNO-TB	4 *(16.7)* 28; 29; 174; 34	15/78 *(19.2)* 42 [26;74]
Final diagnosis		
PCD confirmed nNO-TB	3 *(12.5)* 28; 34; 45	12 *(15.2)* 42 [25;65]
PCD excluded nNO-TB	19 *(79.2)* n = 18, 228 [105;395]	61 *(77.2)* 275 [159;382]
Pending cases nNO-TB	2 *(8.3)* 29; 174	4 *(5.1)* 25; 254;114; 86
Unconcluded cases nNO-TB	0 *(-)*	2 *(2.5)* 33; 56

nNO-TB: nasal Nitric Oxide measurement performed during tidal breathing; nNO-ER: nasal Nitric Oxide measurement performed during expiration against a resistance; TEM: transmission electron microscopy on nasal or bronchial biopsy

*: percentages are related to the population defined in the top cell of each column except for genetic study outcome in children with only nNO-TB result because of missing data (the total number of patients with a known outcome is the denominator)

#: nNO-TB value at inclusion for the children of the cell. The number of measurements is equal to the number of children displayed in the cell, or otherwise specified left of the nNO-TB values. Series of 4 children or less are given *in extenso*

Table E4 – Values of nasal NO changing from above to under the threshold after subtracting ambient NO in 16 tests performed in 15 children

Patients	**AGE**	NOamb	nNO-ER	nNO-ER minus NOamb	nNO-BH	nNO-BH minus NOamb	nNO-TB	nNO-TB minus NOamb
1 ppb	**6.8**	**17.3**			**276**	**258.7**		
2 ppb	**14.2**	**70**	349	279	**327**	**257**	**162**	**92**
3 ppb	**14.7**	**13**	255.2	242.2	**286.2**	**273.2**	**133.6**	**120.6**
4 ppb	**5.7**	**10**	190	180	178	168	**139**	**129**
5 ppb	**5.7**	**45**	142	97	140	95	**136**	**91**
5 ppb	*5.8*	*0.7*					*155.5*	*154.8*
6 ppb	**6.8**	**17**	**290**	**273**	270	253	**139**	**122**
7 ppb	**0.3**	**42**					**68**	**26**
7 ppb	*1.0*	*5*					*121.4*	*116.4*
7 ppb	*3.3*	*5*					*31.48*	*26.5*
7 ppb	*3.9*	*0*					*211*	*211*
8 ppb	**8.1**	**78**			224	146	**205.3**	**127.3**

8 ppb	*8.9*	*17*	*154*	*137*	*143*	*126*	*113.8*	*96.8*
9 ppb	**6.2**	**42**	132	90			**135**	**93**
10 ppb	**5.9**	**18**	**289**	**271**	319	301	187	169
11 ppb	**3.6**	**10.5**					**134**	**123.5**
11 ppb	*4.6*	*20*					*383*	*363*
12 ppb	*3.1*	*30*					*125.6*	*95.6*
12 ppb	**4.1**	**25**					**154.7**	**129.7**
13 ppb	**13.4**	**20**	205	185			**149.8**	**129.8**
13 ppb $nL.min^{-1}$	*13.9*	*24*	*868.8*	*844.8*				
14 ppb	**9.1**	**40**			252	212	**143**	**103**
15 ppb	**6.1**	**6.2**	**277**	**270.8**			208	201.8

NOamb: ambient NO; nNO: nasal nitric oxide; ER: expiration against a resistance; BH breath hold; TB Tidal Breathing

Values in bold are those who changed from above to under the threshold (274 ppb for ER or BH methods, and 133 ppb for TB method) values in italic are those recorded in the same patient on another occasion (before or after). Patients 5, 7, 8, 11, 12, 13 were tested at least twice.

E-Figures

Figure E1 – Standard Of Procedure used since 2015 at Armand Trousseau Hospital

ER: Expiration against resistance method; BH breath hold method; TB: Tidal Breathing method

Figure E2 – Years of measurement distribution of the 454 files included in the study

Figure E3 – Distribution of ambient NO values measured before 449 tests

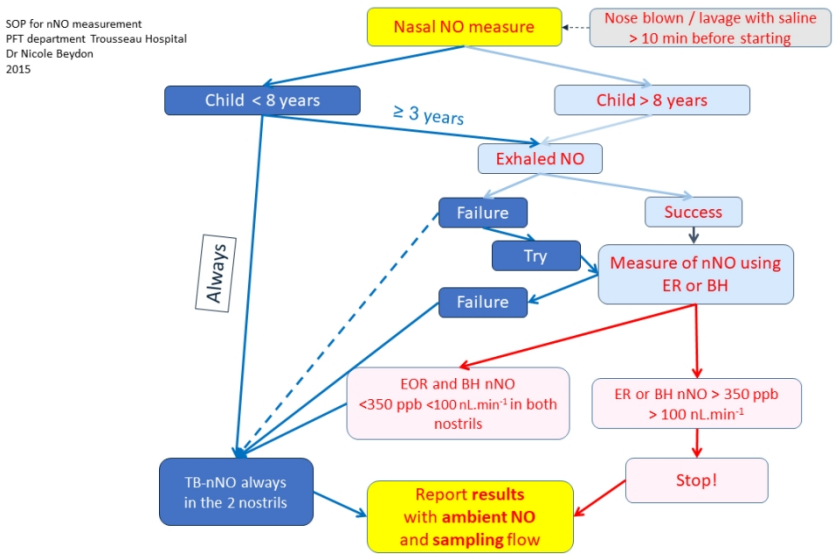
Figures 4 – Difference between two successive measurements of nasal nitric Oxide obtained using the same method according to the delay between the measures

Figure 4a – Difference between two successive measurements of nasal nitric Oxide obtained using the Tidal Breathing method according to the delay between the two measures in 44 children

nNO-TB: nasal Nitric Oxide measured using the Tidal Breathing method

Figure 4b – Difference between two successive measurements of nasal nitric Oxide obtained using the Expiration against a Resistance method according to the delay between the two measures in 26 children

nNO-ER: nasal Nitric Oxide measured using the Expiration against a Resistance method



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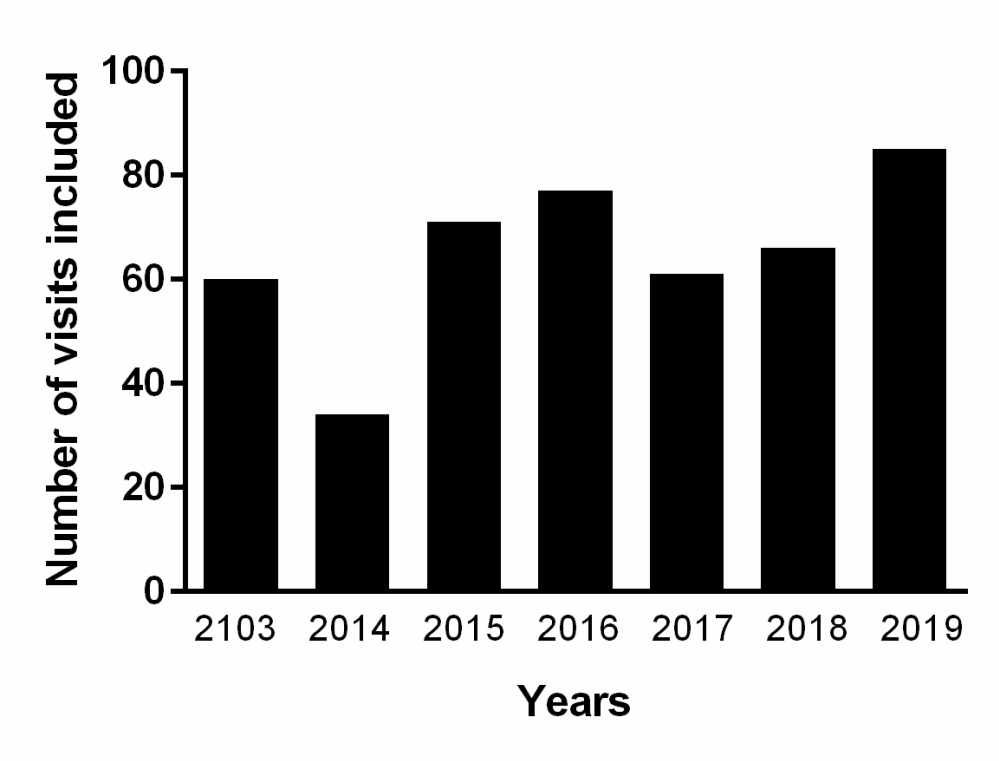

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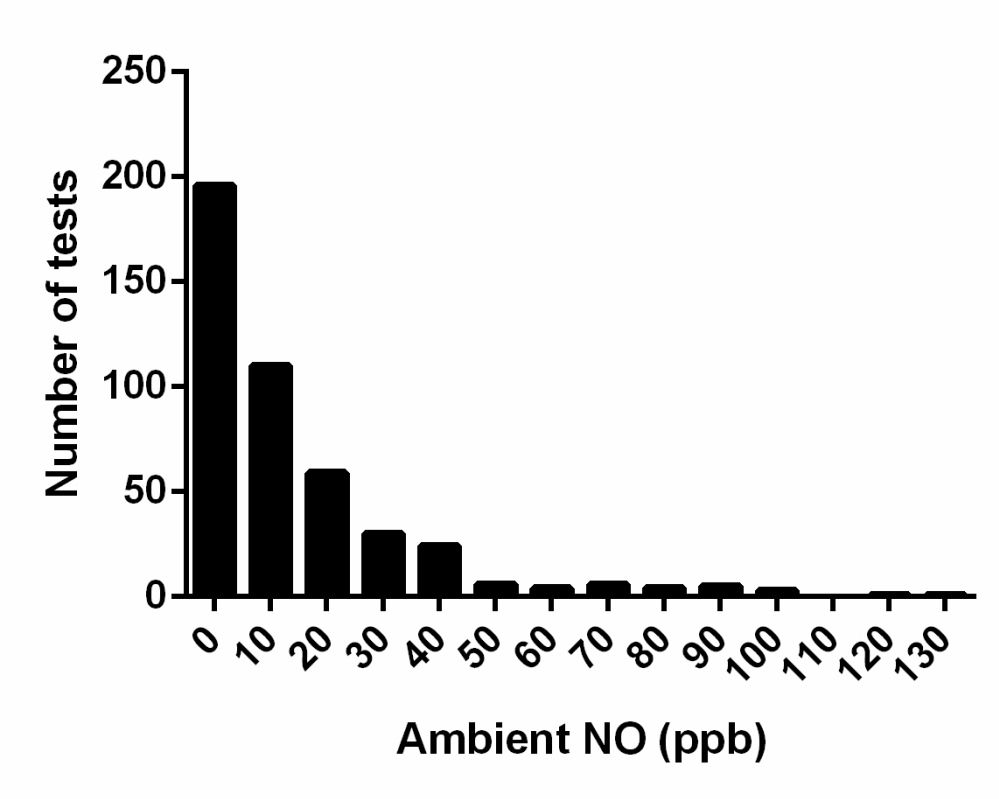

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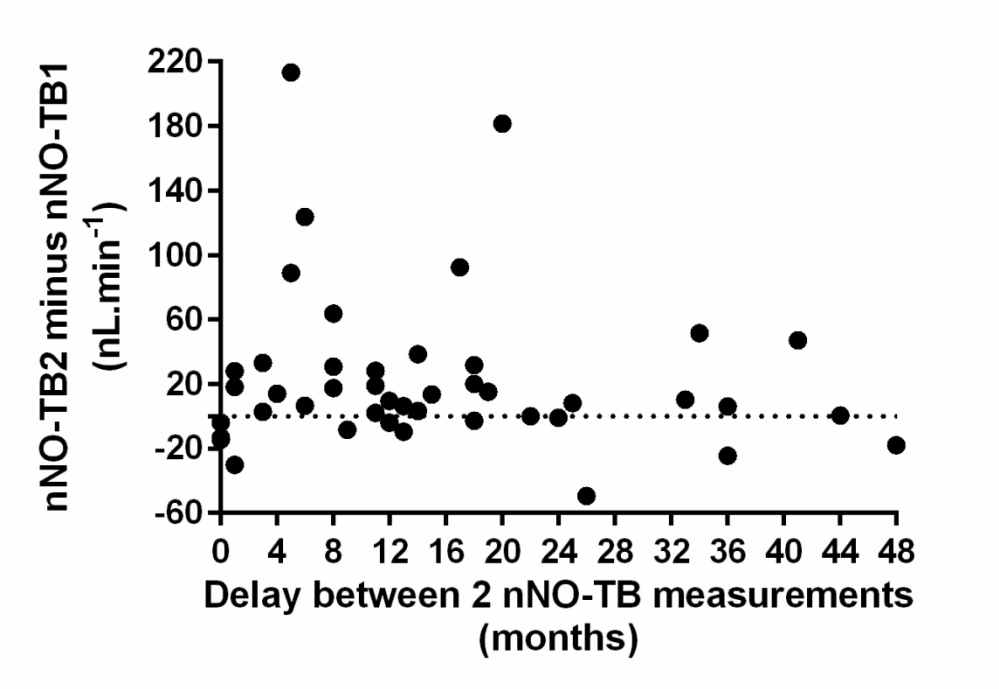

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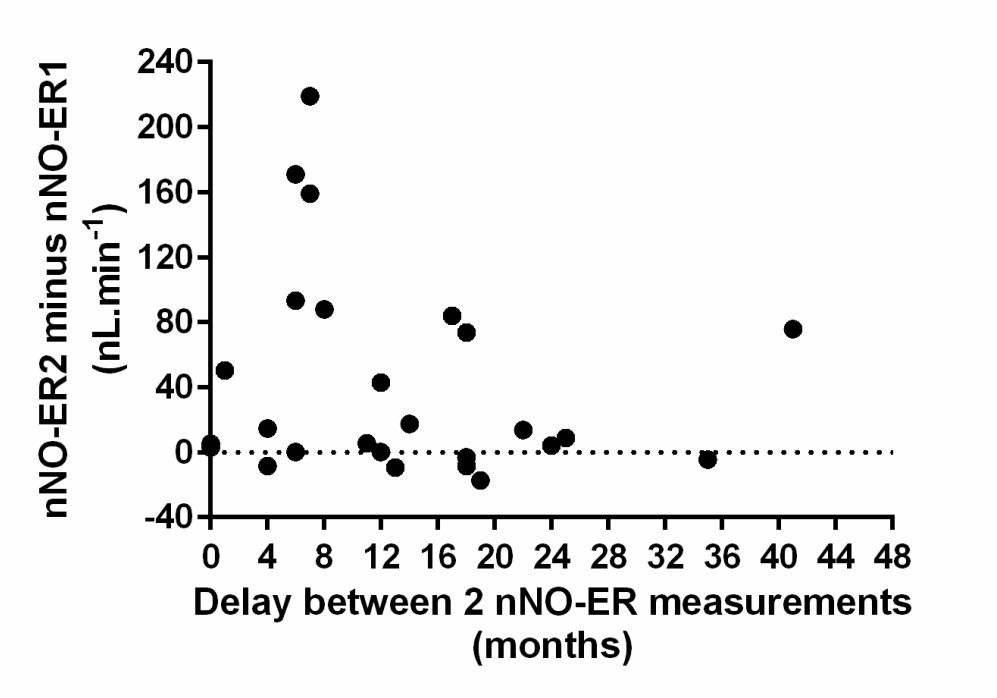

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