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**HIGH NASAL NITRIC OXIDE, CILIA ANALYSES AND GENOTYPES IN A
RETROSPECTIVE COHORT OF CHILDREN WITH PRIMARY CILIARY
DYSKINESIA**

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All authors approved the final version submitted and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Abstract

Rationale: While children with primary ciliary dyskinesia (PCD) typically have low nasal nitric oxide (nNO), some children with indisputable PCD may have unexplained high nNO levels.

Objectives: To look for relationships between nNO measures and genetic findings (and cilia motility or ultrastructure when available) in PCD children with a known genotype.

Methods: We studied retrospectively 73 PCD children (median (range) age 9.5 (2.1 to 18.2) years). nNO was the mean value of a plateau reached while velum was closed (nNO-VC, threshold 77 nL.min⁻¹), or calculated as the average of 5 peaks obtained during tidal breathing (nNO-TB, threshold 40 nL.min⁻¹). Cilia beat was classified either as motile (including dyskinetic pattern) or immotile depending on whether motility was present or absent in all cilia, or as a mixture of motile and immotile cilia. Genotypes were classified as: pathogenic mutations in a gene known to be associated with high nNO (mild genotype); bi-allelic truncating mutations in other genes (severe mutations); putative hypomorphic pathogenic mutation (missense, single amino-acid deletion or moderate splicing mutations) in at least one allele thought to be possibly associated with a residual production of a functional protein.

Main Results: nNO was above the discriminant threshold in 16/73 (21.9%) children (11 nNO-VC and 5 nNO-TB). High nNO was less frequent in children with severe mutations (2/42) than in those with mild genotypes (7/10) or at least one hypomorphic mutation (7/21) ($P < 0.0001$). Median [IQR] nNO-VC values (n=60) were significantly different in the three genotypic groups: severe mutations 18 [10;26] nL.min⁻¹ (n=36), possible residual functional protein production (putative hypomorphic mutations) 23 [16;68] nL.min⁻¹ (n=17), and mild genotypes 139 [57;216] nL.min⁻¹ (n=7); $P=0.0002$.

The higher the cilia motility the higher the nNO-VC (16 [10;23], 23 [17;56], and 78 [45;93] nL.min⁻¹ in patients with respectively immotile, dyskinetic motile/immotile, and dyskinetic motile cilia; $P < 0.0001$), while nNO values were scattered across different ultrastructure defects ($P = 0.07$).

Conclusions: In PCD children, high nNO values were linked not only to specific genes and but also to potentially hypomorphic mutations in other genes (with possible functional protein production). nNO values increased with the proportion of motile cilia.

Word count: 349

Introduction

Primary ciliary dyskinesia (PCD) is an inherited disorder affecting motile cilia, which results in mucociliary clearance impairment. Patients usually suffer from lifelong symptoms of chronic wet cough and rhinosinusitis due to recurrent respiratory infections leading to bronchiectasis (1). Early diagnosis is important to slow down pulmonary deterioration and improve quality of life. Although the mechanism is unknown, patients with PCD are commonly reported to have low nasal nitric oxide (nNO) output, which represents a highly sensitive and specific test for PCD diagnosis (2,3). Both the European Respiratory Society task force and the American Thoracic Society guidelines recently recommended it as a first-line test in patients with symptoms suggestive of PCD (3,4). When nNO levels are low, transmission electronic microscopy (TEM) assessment of the ciliary axonemes and/or genetic analyses are required to confirm the diagnosis of PCD.

However, patients with confirmed PCD and high nNO values (above the discriminant threshold) have rarely been reported (3). This phenotype has been reported in patients with pathogenic mutations in three genes known to be associated with a mild ciliary phenotype (*RSPH1*, *CCDC103* p.His154Pro, *DNAH9*) (5–8), or in sporadic cases with indisputable causal mutations (*GAS2L2*, *FOXJ1*, *CFAP221*, *NEK10*, *TTC12*) (9–13). Of note, specific TEM defects (inner dynein arm defect with microtubular disorganization) have been related to more severe clinical outcomes (14,15), whereas no relationships between TEM results and low or high nNO values were reported (16,17).

Thus, as with normal TEM, normal nNO values do not exclude the diagnosis of PCD in subjects with a medical history and/or ciliary beat analyses in favor of this diagnosis. In these patients with normal nNO and TEM, identification of bi-allelic loss-of-function

mutations can confirm the diagnosis of PCD, whereas the absence of such mutations cannot exclude it.

We hypothesized that some PCD mutations can result in the presence of a functional residual protein which could be responsible for persistent nNO production and ciliary motility. Given that cilia ultrastructure defects do not always preclude cilia motility (eg. *DNAH11* associated with normal ultrastructure and severe impairment in cilia motility), we also hypothesized that cilia ultrastructure would not always reflect persistent nNO production. The main aim of this study was to unravel the relationships between high and low levels of nNO and the expected molecular consequences of pathogenic mutations detected in PCD patients. Secondary outcomes were to assess the possible relationships between high and low levels of nNO and cilia motility or ultrastructure pattern.

Methods

Subjects

We performed a single extraction, from the database of the molecular genetics department of our tertiary hospital (Armand Trousseau University Hospital, Paris, France), of all files of children with a PCD diagnosis and a causal genotype (bi-allelic pathogenic mutations in a known PCD gene, hemizygous mutation in *RPGR* or *PIH1D3*, heterozygous mutation in *FOXJ1*). We then crossed this list with the list of children in whom a nNO measurement was performed in Armand Trousseau Hospital or Necker Enfants-Malades Hospital following a common Standard Operating Procedure (SOP) (18,19). Written informed consent was obtained from all parents and the study was approved by the Comité de Protection des Personnes (CPP) Ile de France III (CPP02748).

Nasal Nitric Oxide Measurement

Nasal NO was measured using equally accurate chemiluminescent NO analysers (NIOX Flex, Aerocrine Solna, Sweden, from 2009 to 2014, in Trousseau hospital, EVA4000, Seres, Aix en Provence, France, from 2009 to 2012 in Necker Enfants-Malades hospital, then CLD 88sp, EcoMedics AG, Duernten, Switzerland, in both hospitals) displaying the real-time NO curve on the screen and sampling the air at a similar rate ($0.3 \text{ L}\cdot\text{min}^{-1}$ for NIOX Flex and EVA4000 machines, and $0.33 \text{ L}\cdot\text{min}^{-1}$, for CLD 88sp)(2). Ambient NO was recorded before each test and NO measurements were performed as previously described in children with no signs of acute respiratory infection, nasal bleeding or malformation (details are available in the Online Supplement) (18). According to our SOP, children were invited to perform a measurement during Tidal Breathing (TB) before trying to perform a measurement using a method involving velum closure (VC) (*ie.* Expiration against a Resistance or Breath Hold). The result of nNO was categorized as high when it was above the discriminating threshold of $77 \text{ nL}\cdot\text{min}^{-1}$ for VC methods (hereafter referred to as nNO-VC) or above $40 \text{ nL}\cdot\text{min}^{-1}$ during TB (hereafter referred to as nNO-TB) in children unable to perform a VC maneuver, otherwise it was qualified as low (2,4,18–20). All children were invited to control the nNO measurement on a second occasion, but this second measurement could be difficult to organize in children living far from our center (20). Spirometry (BodyBox, Medisoft, Sorinnes, Belgium) was performed according to current recommendations at the time of the study.

Ciliary beat analyses

Ciliary motility was evaluated within 6 hours after airway brushing. The sample was immediately immersed in culture medium and analysed either by optic microscopy or, since 2012, by High Speed Video Microscopy (HSVM), as previously described (21).

Ciliary beat pattern was evaluated in at least 10 different fields and divided into three categories according to the proportion of dyskinetic motile/immotile cilia: total immotility (0% of motile cilia), coexistence of dyskinetic motile and immotile cilia (regardless of the proportion of motile versus immotile cilia), and dyskinetic motile cilia (100% of motile cilia, ie. no immotile cilia observed in the analysed fields). (details are available in the Online Supplement)

Ultrastructural analyses

The ciliary ultrastructure was analysed by TEM on airway biopsies as previously described (22). Results were expressed as the percentage of abnormal cilia among the analysed cilia (≥ 50 per patient) and as the main ultrastructural abnormality among the abnormal cilia. The TEM conclusions were categorized in five classes: combined outer/inner dynein arms defect (ODA+IDA), outer dynein arm defect (ODA), inner dynein arm defect with microtubular disorganisation (IDA/MTD), abnormal central complex defect (CC), normal ultrastructure (normal TEM) (3).

Genetic analyses

Next-generation sequencing technique is detailed in the Online Supplement.

Genotypes were categorized in three groups. The first group (referred to as mild genotypes) corresponded to the genotypes for which high nNO level has been reported in a significant number of patients, together with mild clinical and/or functional PCD features (i.e. *RSPH1* and the p.His154Pro mutation in *CCDC103*-) (5,6). For the other implicated genes, the

genotypes were further categorized in two groups, according to the type of pathogenic mutations. The second group (referred to as severe mutations) corresponded to bi-allelic truncating mutations in other genes. The third one (referred to as potentially mild mutations) gathered genotypes with at least one potentially hypomorphic mutation. In all patients, we assumed the potential production of a residual amount of a functional protein according to mutations. Severe mutations were nonsense, frameshift or severe splice defects that would lead to the absence of protein or to a truncated non-functional protein production. Potentially mild mutations included missense mutations, single amino-acid deletions, and moderate splice defects (SpliceAI prediction score between 0.20 and 0.80) that could correspond to a small amount of full-length protein. Homozygous or compound heterozygous genotypes for severe mutations were assumed to lead to a complete loss in protein production, whereas the presence of one or two allele(s) with a potentially mild mutation may enable the processing of a residual and/or functional protein.

In parallel, the pathogenicity of all mutations has been assessed following the American College of Medical Genetics guidelines (23). Briefly, the pathogenicity was assessed based on previous description in other patients, allelic frequency in the general population (gnomAD), potential effect on splicing (SpliceAI) and CADD pathogenicity prediction score. For missense mutations, conservation in vertebrates (100 vertebrates, UCSC) has also been verified. Additionally, coherence between the mutated genes and the TEM outcome was checked for. Of note, for rare missense mutations that had never been previously described in patients, we did not consider coherent TEM ultrastructure as a proof for pathogenicity, and therefore, we conservatively classified them as variants of uncertain significance.

Statistical analyses

Quantitative variables are reported as mean (Standard Deviation (SD)) or median [1st; 3rd quartiles] and qualitative variables as a number (percentage). Between-group comparisons were performed using t-tests, Mann-Whitney or Kruskal-Wallis tests, and Fisher exact test, as appropriate. Due to the difference in nNO values obtained using the VC or TB method, only nNO-VC results were used as continuous values in the statistical analyses, while both nNO results (nNO-VC and nNO-TB) were included in qualitative analyses. Forced expiratory volume in 1s (FEV₁) was expressed as z-score using the GLI reference values (24). The *P*-value confirmed a statistical relationship when <0.05 (two-sided). Analysis was performed using GraphPad Prism (version 6.01)

The multidimensional grouping of patients according to previously defined groups of genotype and nNO level, cilia motility and ultrastructure, has been studied through a principal component analysis using the ClustVis tool (25) (details on the construction of the figure are available in the Online Supplement).

Results

We included 73 PCD children with a median [IQR] (range) age at nasal NO measurement of 9.5 [6.4 to 13.6] (2.1 to 18) years. The FEV₁ was measured in 67 children (median [IQR] age 9.2 [7.1 ; 12.6] years at first measurement) and repeated in 59 children (16.5 [11.3 ; 18.3] years at last measurement) (Table 1). The last FEV₁ was lower than the first one in all study children and in the group of children with low nNO, but not in the group of children with high nNO value. The median slope of FEV₁ decrease was nearly zero in children with high nNO and slightly more negative in children with low nNO (*P* = 0.10).

The majority of children had a well characterized clinical phenotype with ciliary investigations available in 70 children (Tables 1, 2 and 3). Most of the children had immotile cilia (n = 53/64; 82.8%, 95%CI: 71.2% to 91.1%) in all cilia (n = 27), or a combination of dyskinetic motile/immotile cilia (n = 26). The main ultrastructural defect concerned ODA (n=35/64; 54.7%, 95%CI: 41.7% to 67.2%), in isolation (n = 19), or combined with an absence of IDA (n = 16), while IDA/MTD, CC and normal TEM accounted for 11 (20%), 7 (12.7%) and 8 (14.5%) cases, respectively. Mild genotypes were identified in 10/73 (13.4%, 95%CI: 6.8% to 23.7%) children (six and four children with mutations in *RSPHI* or the p.His154Pro *CCDC103* mutation, respectively), while two severe mutations in other PCD genes were identified in 42/73 (57.5%, 95%CI: 45.4% to 69.0%) patients (see details and Table E1 in the Online Supplement). According to the genotype, a mild genotype or a possible production of a functional protein was considered in 31/73 (42.5%, 95%CI: 30.1% to 54.6%) children. All putative hypomorphic mutations were in genes coherent with the TEM defect.

The distribution of all nNO-VC and nNO-TB measurements are shown in Figure E1. Sixty children had nNO-VC results and 13 children performed only nNO-TB measurements (of whom three were less than 4 years of age, see Online Supplement). Of these 13 children with only nNO-TB results, five were classified as having high nNO value. Three had a nNO-TB > 77 nL.min⁻¹ and two between the nNO-TB threshold we previously established (40 nL.min⁻¹) and 77 nL.min⁻¹ (18,19) (63 nL.min⁻¹ in a 3.9-year-old child carrying a *RSPHI* mutation, and 49 nL.min⁻¹ in a 14-year-old child carrying a *DNAH5* homozygous missense mutation introducing a Proline, and showing short or absent ODAs) (Table E1).

For the seven children tested with an ambient NO > 10 ppb, the classification of their nNO would not be changed from high to low should the ambient NO be subtracted from their results. Overall, 16/73 (21.9%, 95% CI: 13.1% to 33.1%) children had a nNO higher than the discriminant threshold (Figure E1 and Table 3).

Of the 33 children seen on a second occasion, 26 confirmed their low nNO value (23 nNO-VC and 3 nNO-TB measurements) and six their high nNO value (5 nNO-VC and 1 nNO-TB measurements). One child with obstructive rhinitis could not perform the second test. nNO-VC values (performed in 60 children) according to ciliary motility, TEM results and genotype severity groups are given in Table 2. nNO-VC values decreased with the proportion of immotile cilia across the three classes of ciliary beat analysis ($P < 0.0001$) (Figure 1) while it was variable across the five ultrastructural patterns ($P = 0.07$) (Figure 2). Lastly, patients with pathogenic mutations in genes linked to a known mild PCD ciliary phenotype had higher nNO-VC values than those with at least one mild pathogenic mutation in other genes; this latter group showing higher nNO-VC than the group with two severe mutations in other genes ($P = 0.0002$) (Figure 3). Children with severe mutations, and therefore an unlikely residual protein production, had lower nNO-VC than the other children of the study ($P = 0.0007$) (Figure 3). The nNO-VC values according to genotypes are shown in Figure E1a.

The distribution of children according to classes of nNO (low or high) and to cilia beat pattern (n=64, $P = 0.002$), TEM findings (n=64, $P = 0.30$), and genotypes (n=73, $P = 0.003$), are given in Table 3.

In the two classes of patients with persistent dyskinetic motile cilia, we observed more patients with high nNO than in patients with no residual motility (12/37 versus 1/27; $P =$

0.005, Table 3). Lastly, when grouping patients with mild genotypes or at least one mild mutation, we observed more patients with high nNO compared to patients who carried two severe mutations in a gene not previously linked to a high nNO (14/31 versus 2/42; $P < 0.0001$, Table 3).

The principal component analysis for the 73 patients including genotype, ciliary defect, motility and NO classes (Figure 4) showed two separate clusters for patients with a mild genotype and those with severe mutations in another gene (distinct 95% confidence ellipses). This indicated that those two groups tended to have discrete features regarding ciliary investigation outcomes and nNO levels. On the other hand, patients with at least one potentially mild pathogenic mutation in another gene were indistinctly scattered across the confidence ellipses of the two other groups.

Discussion

While nNO measurement is considered a highly sensitive and specific tool to screen for PCD disease, we measured it higher than the discriminant threshold in 16 out of 73 children (21.9%) with a PCD diagnosis asserted by molecular analyses (3,4,26,27).

Although not all children were able to perform a VC maneuver or a second measurement on another occasion, we believe we have accurately classified children with high and low nNO levels. Against the recommendations released in a technical standard published after our study (20), we used a maximum 10% variation in the measured range of the nNO plateau because 5% variation can be difficult to achieve when nNO level is very low; we never measured nNO in crying children because it is impossible to determine whether the velum is closed or not at that time. For the rest, our SOP fit with that of this technical

standard, in which TB is not the gold standard but a surrogate measure of NO usable from the age of 2 years. With this method (TB), nNO was indisputably high in four children, and may be discussed in one adolescent who had a nNO-TB value of 49 nL.min⁻¹ (threshold 40 nL.min⁻¹) (18,19). This patient's evaluation would not have changed the results of the between-group comparisons of nNO values, which were solely based on nNO-VC measurements. Unfortunately, nNO in this adolescent could not be controlled, but in the 32 children who we measured a second time, the classification of nNO was consistent regardless of the method used to measure nNO. Lastly, considering that the only way to erroneously increase nNO value would be to perform the measurement in a setting where ambient NO is high, we checked that after subtracting the ambient NO, all nNO results remained on the same side of the threshold (inducing no false high nNO value in our study). The sensitivity of nNO to discriminate for PCD diagnosis was reported to be 89% to 100%, but lower sensitivities such as 74% and 86.1% were reported in two studies, one of which lacked genetic analyses (28,29). The high proportion of children with nNO values above the threshold could reflect our activity as a reference PCD Centre to which difficult cases are referred.

A nNO level higher than the discriminant threshold has already been reported in patients in whom ODA and axonemal organisation are usually spared, either in sporadic cases in original publications of newly identified PCD genes (such as *GAS2L2*, *FOXJ1*, *CFAP221*, *NEK10*, *TTC12*), or for some specific PCD genes (i.e. *RSPH1*, *DNAH9* and *CCDC103* p.His154Pro) responsible for a mild disease in multiple cases (5–13). For patients with pathogenic mutations in these three latter genes, a less severe functional (and clinical for

RSPH1) phenotype compared to that of patients with pathogenic mutations in other PCD genes, has been demonstrated (5–8).

Among the patients with high nNO, 14/16 had a potentially mild genotype: seven had mild genotypes (*RSPH1* n=5, *CCDC103* p.His154Pro n=2), one had a mutation in *FOXJ1* (autosomal dominant transmission with one normal allele), and six had mutations in another gene but a potentially mild mutation in one allele. The latter six patients carried at least one missense mutation in a gene linked to the absence of ODA (*DNAH5* n=4, *SPAG1* n=1, *DNAAF5* n=1). Those three genes are not usually associated with a mild disease or high nNO. By contrast, among the 13 other patients with *DNAH5* or *SPAG1* mutations and a low nNO, only two were heterozygous for a *DNAH5* missense mutation. The remaining 2/16 patients with high nNO carried mutations leading to a premature stop codon in *TTC12* (isolated absence of IDA), and in *RSPH3* (encoding a major component of the stalk in radial spokes), both showing spared ODA.

We can reason that when mutations lead to a premature stop codon in genes coding for axonemal structure crucial for cilia motility (such as dynein arms), a low nNO is expected. On the other hand, some pathogenic hypomorphic mutations would underlie nNO above the diagnostic threshold thanks to more preserved cilia motility (30). Regarding nNO levels in patients with putative hypomorphic mutations, 5/13 of the patients with at least one missense had high nNO, whereas 1/6 of the patients with at least one potentially moderate splice mutation had high nNO. Given the small number of patients, predicting which potentially hypomorphic mutation would correspond to a high nNO still seems hazardous. Further analyses in large groups of patients, e.g. unsupervised approaches, may help sorting those potentially hypomorphic mutations.

The group of children carrying mutations in *DNAH5* is a particular group because it represents a large proportion of children in the study (16/73) who could have high or low nNO. All patients with *DNAH5* mutations and a high nNO (n=4) had typical clinical PCD features including early onset sinopulmonary symptoms and bronchiectasis, but no situs inversus. Those four patients carried at least one potentially mild allele whereas this was the case for only 2/12 patients with *DNAH5* pathogenic mutation and a low nNO. Of note, 3/4 of the patients with *DNAH5* mutations and a high nNO had a proportion of ODA truncation, which was not the case in the 12 patients with *DNAH5* mutations and a low nNO. Residual targeting of *DNAH5* to the proximal region of the axonem has already been demonstrated in patients carrying one hypomorphic mutation (31). In summary, some patients with *DNAH5* potentially mild mutations are likely to produce a residual amount of protein that would result in the existence of ODA remnants and a higher level of nNO, thanks to better cilia motility. Unfortunately, motility data was only available for one of these patients with high nNO (motile cilia).

Pathogenic mutations in each PCD gene are responsible for a specific ultrastructural defect secondary to the absence or loss of function of a protein. Similarly, each type of ultrastructural defect usually leads to the same abnormal beating pattern with numerous immotile cilia in case of dynein arm defects and almost no immotile cilia in case of CC defects (30).

In line with these known relationships, high nNO was more frequently measured in patients with persistent dyskinetic motile cilia compare to patients with total immotility (32.4% versus 3.7%, Table 3). The significant difference we found according to the degree of cilia motility is confirmed by a recent study in which nNO was significantly higher when the

beat frequency was above 2 Hz compared to when it was below 2 Hz (27). Even though the precise mechanism responsible for the decrease in nNO production in PCD remains unknown, the possible role of shear stress caused by cilia beating in the activation of cilia NO synthase could be part of the explanation (32–34). In addition, there was no clear differences in nNO values across the five ultrastructure classes. A majority of patients (55.5%) with CC defects had high nNO, while when TEM showed defects in dynein arms (ODA+IDA, ODA, IDA/MTD), most of the patients (78.3%) exhibited low nNO values (Table 3). Interestingly, all patients with normal TEM due to mutations in *DNAH11* had low nNO, probably because *DNAH11* codes for a proximal ODA heavy chain, and its mutations are responsible for the loss of ODAs in the proximal part of the axoneme which can only be detected by tomoelectromicroscopy (35) (Figure E1). This may partly explain the lack of significance of the difference between nNO levels measured in children with normal TEM or DA TEM defects (27).

The clustering approach underlined this phenotype-genotype link by delineating two discrete clusters for patients with mild genotypes and those with two severe mutations in another gene, respectively (Figure 4). Only two patients from the latter group fell outside the 95% confidence interval ellipse and appeared closer to patients with mild genotypes: one was homozygote for a *TTC12* nonsense mutation (described in (13)) and one was homozygote for a *RSPH3* splice mutation. This suggests that mutations in *TTC12* and *RSPH3* – respectively responsible for IDA and radial spoke defects while preserving ODA – could be associated with a milder phenotype in terms of ciliary features and nNO production, but further studies are warranted, especially for *RSPH3* mutations. On the other hand, principal component analysis did not show any obvious clustering for patients who

carry a potentially mild pathogenic mutation on at least one allele. This is probably due to our current inability to accurately predict the functional consequences of missense mutations and variations that do not fully disrupt splice sites.

Our study has some limitations. The study design biased the exact proportion of PCD children with high nNO since we limited inclusion to patients with both genetic diagnosis and nNO measurement available. Therefore, we cannot infer sensitivity/specificity from our data (details in the Online Supplement). We cannot comment on the specific clinical signs that prompted us to pursue PCD workup, but we found no difference between the clinical presentation of children with high or low nNO (Table 1). In addition, we can report that the clinical signs on which we rely to decide whether or not to continue the evaluation in difficult cases are in agreement with those included in the Official American Thoracic Society Clinical Practice Guideline (4). Of note, clinical characteristics of the studied children were similar to those described in previous PCD series, except for the frequency of situs anomalies, which was lower than usual. Children with problematic diagnosis are more likely to be referred to our centre where an extensive TEM and molecular analyses can be performed, improving the identification of atypical PCD without laterality defect.

In conclusion, we highlight the link between high nNO in PCD patients and residual cilia motility, together with specific genotypes, some of which being suggested by the TEM defects. These genotypes encompass pathogenic mutations in *RSPH1*, *CCDC103* (p.His154Pro), *TTC12*, *FOXJ1*, and genotypes implicating other genes (such as *DNAH5*) with at least one allele that may not lead to a complete loss of function (hypomorphic mutations). Whether patients with high nNO value present a mild course of PCD disease

cannot be demonstrated from our retrospective study. Other multicenter prospective studies carried out in large populations would make it possible to better evaluate the clinical characteristics and the evolution of these patients.

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Links

CADD <https://cadd.gs.washington.edu/>

ClustVis <https://biit.cs.ut.ee/clustvis/>

SpliceAI <https://github.com/Illumina/SpliceAI>

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Table 1 – Clinical characteristics in 73 study children with primary ciliary dyskinesia, according to nasal NO levels

| | MD | All children | MD | Children with high nasal NO | MD | Children with low nasal NO |
|--------------------------|-----------|-------------------------|-----------|------------------------------------|-----------|-----------------------------------|
| Number of children | | 73 | | 16 | | 57 |
| Sex M/F | | 45 (61.6%) / 28 (38.4%) | | 9 (56.2%) / 7 (43.8%) | | 36 (63.1%) / 21 (36.9%) |
| Age at diagnosis (years) | | 7.3 (4.4) | | 6.5 (3.9) | | 7.5 (4.5) |
| Consanguinity | 1 | 23 (31.9%) | 1 | 3 (20%) | | 20 (35.1%) |
| Situs anomalies | | 25 (34.2%) | | 5 (31.2%) | | 20 (35.1%) |
| Neonatal chest symptoms | 2 | 48 (67.6%) | | 9 (56.2%) | 2 | 39 (70.9%) |
| Congenital heart disease | | 4 (5.5%) | | 0 (0%) | | 4 (7.0%) |
| Chronic wet cough | | 72 (98.6%) | | 15 (93.7%) | | 57 (100%) |
| Bronchiectasis | | 44 (60.3%) | | 7 (43.7%) | | 37 (64.9%) |

| | | | | | | |
|--|----|-------------------------|---|-------------------------|---|-------------------------|
| <i>Pseudomonas aeruginosa</i> infection (ever) | | 13 (17.8%) | | 1 (6.2%) | | 12 (21.0%) |
| Chronic rhinitis | | 63 (86.3%) | | 11 (68.7%) | | 52 (91.2%) |
| Chronic otitis media | | 71 (97.3%) | | 15 (93.7%) | | 56 (98.2%) |
| Hearing loss | | 24 (32.9%) | | 7 (43.7%) | | 17 (29.8%) |
| First FEV ₁ (z-score) | 6 | -1.23 (1.52) | 4 | -1.31 (1.54) | 2 | -1.21 (1.53) |
| Last FEV ₁ (z-score) | 14 | -1.58 (1.56)* | 6 | -1.24 (1.82) | 8 | -1.65 (1.51)* |
| Slope of FEV ₁ (z-score/year) | 14 | -0.11 [-0.21 ; 0.03] | 6 | -0.04 [-0.13 ; 0.23] | 8 | -0.12 [-0.22 ; 0.02] |

Results are number (*percentage*), mean (SD) or median [1st; 3rd] quartiles

MD: missing data; FEV₁: Forced Expiratory Volume in 1 s

* Last FEV₁ lower than first FEV₁ in all the study patients ($P = 0.01$) and in patients with low nasal NO ($P = 0.0008$)

Table 2 – Distribution of ciliary beat patterns, transmission electronic microscopy findings, genotype severity and nasal NO measured velum closed in 60 children with primary ciliary dyskinesia

| | MD | Number of children | nNO-VC (nL.min ⁻¹) |
|---|----------|--------------------|--------------------------------|
| Ciliary motility | 4 | 56 | |
| Immotile cilia | | 24 (42.9%) | 16 [10;23] |
| Dyskinetic and immotile cilia | | 21 (37.5%) | 23 [17;56] |
| Dyskinetic cilia | | 11 (19.6%) | 78 [45;93] |
| TEM defect | 1 | 55 | |
| ODA+IDA | | 14 (25.5%) | 25 [17;81] |
| ODA | | 15 (27.3%) | 17 [10;23] |
| IDA/MTD | | 11 (20%) | 19 [12;54] |
| CC | | 7 (12.7%) | 45 [35;139] |
| Normal TEM | | 8 (14.5%) | 24 [10;39] |
| Genotype severity | 0 | 60 | |
| Mild genotypes * | | 7 (11.7%) | 139 [57;216] |
| Other genes with ≥ 1 potentially mild mutation [†] | | 17 (28.3%) | 23 [16;68] |
| <i>Of which 1 mild mutation</i> | | 8 | 56 [19;95] |
| <i> 2 mild mutations</i> | | 9 | 20 [16;36] |
| Other genes with 2 severe mutations [§] | | 36 (60%) | 18 [10;26] |

Results are number (*percentage*) or median and [IQR].

MD: missing data; NO-VC: Nitric Oxide measured with velum closed; TEM: transmission electron microscopy; ODA+IDA: absence of ODA and IDA; ODA: Outer Dynein Arm defect; IDA/MTD: Inner Dynein Arm defect with microtubular disorganisation; CC: Central Complex defect; normal TEM: normal ultrastructure.

*: Mild genotypes correspond to *RSPH1*, and to the *CCDC103* p.His154Pro mutation.

† group of children with a possible residual functional protein due to the presence of 1 or 2 mild mutations in other genes

§: group of children in which a residual functional protein is unlikely due to the presence of 2 severe mutations in other genes

Table 3 – Distribution of ciliary beat patterns, transmission electronic microscopy findings, and genotype severity according to low or high levels of nasal NO in 73 children with primary ciliary dyskinesia

| | MD | Number of children | Low nNO | High nNO | P-value |
|--|----------|--------------------|--------------|--------------|---------|
| Ciliary motility | 9 | 64 | | | 0.002 |
| Immotile cilia | | 27 | 26 (40.6%) | 1 (1.5%) | |
| Dyskinetic and immotile cilia | | 26 | 20 (31.3%) | 6 (9.4%) | |
| Dyskinetic cilia | | 11 | 5 (7.8%) | 6 (9.4%) | |
| TEM defect | 9 | 64 | | | 0.30 |
| ODA+IDA | | 16 | 12 (18.8%) | 4 (6.2%) | |
| ODA | | 19 | 15 (23.4%) | 4 (6.2%) | |
| IDA/MTD | | 11 | 9 (14.1%) | 2 (3.2%) | |
| CC | | 9 | 4 (6.2%) | 5 (7.8%) | |
| Normal TEM | | 9 | 8 (12.5%) | 1 (1.6%) | |
| Genotype severity | 0 | 73 | | | |
| Mild genotypes [*] | | 10 | 3 (4.1%) | 7 (9.6%) | |
| Other genes with ≥ 1 potentially mild mutation [†] | | 21 | 14 (19.2%) | 7 (9.6%) | |
| <i>Of which 1 mild mutation</i> | | 10 | 5/21 (23.8%) | 5/21 (23.8%) | |
| <i> 2 mild mutations</i> | | 11 | 9/21 (42.9%) | 2/21 (9.5%) | |
| Other genes with 2 severe mutations [§] | | 42 | 40 (54.8%) | 2 (2.7%) | |

Results are number (*percentage*).

MD: missing data; nNO: nasal Nitric Oxide measured while velum closed or during tidal breathing; TEM: transmission electron microscopy; ODA+IDA: absence of ODA and IDA; ODA: Outer Dynein Arm defect; IDA/MTD: Inner Dynein Arm defect with microtubular disorganisation; CC: Central Complex defect; normal EM: normal ultrastructure.

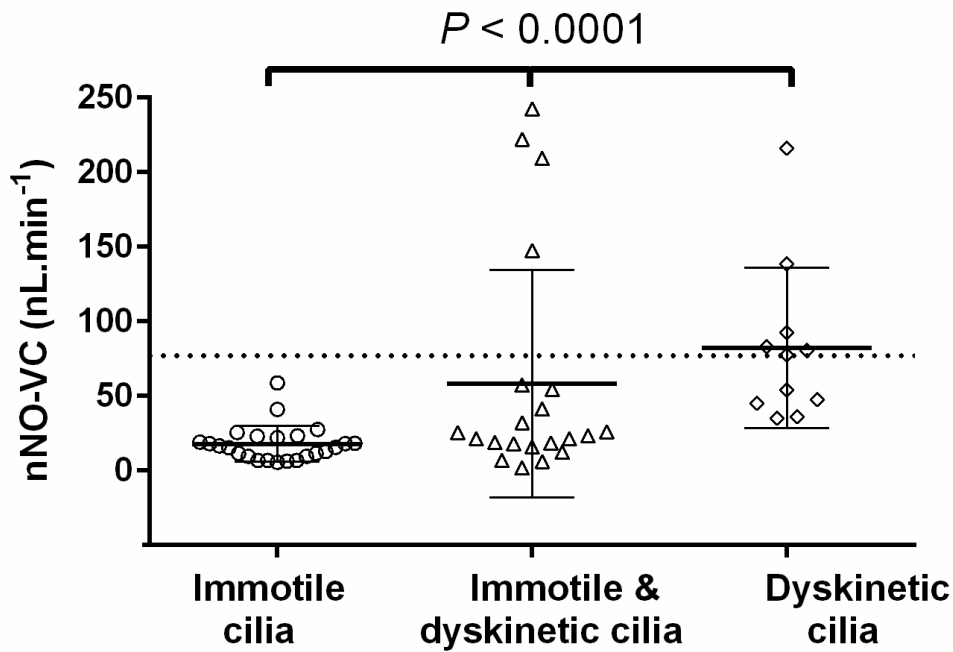
*: Mild genotypes correspond to *RSPH1*, and to the *CCDC103* p.His154Pro mutation.

† group of children with a possible residual functional protein due to the presence of 1 or 2 potentially mild mutations in other genes

§: group of children in which a residual functional protein is unlikely due to the presence of 2 severe mutations in other genes

Figures

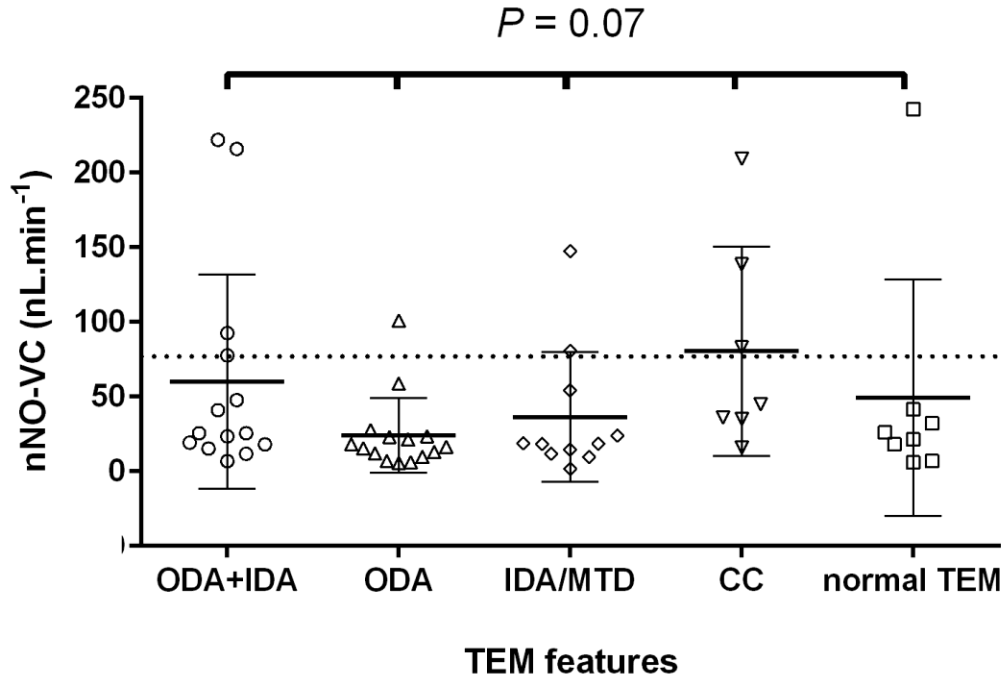
Figure 1 – Distribution of nasal Nitric Oxide measured with velum closed according to cilia motility measured in 56 children



Dotted line is the discriminant threshold (77 nL.min⁻¹).

Circles are values in children with immotile cilia, triangles are values in children with both dyskinetic motile and immotile cilia, and diamonds are values in children with dyskinetic motile cilia.

Figure 2 – Distribution of nasal Nitric Oxide (nNO) measured with velum closed according to cilia ultrastructure defect in 55 children

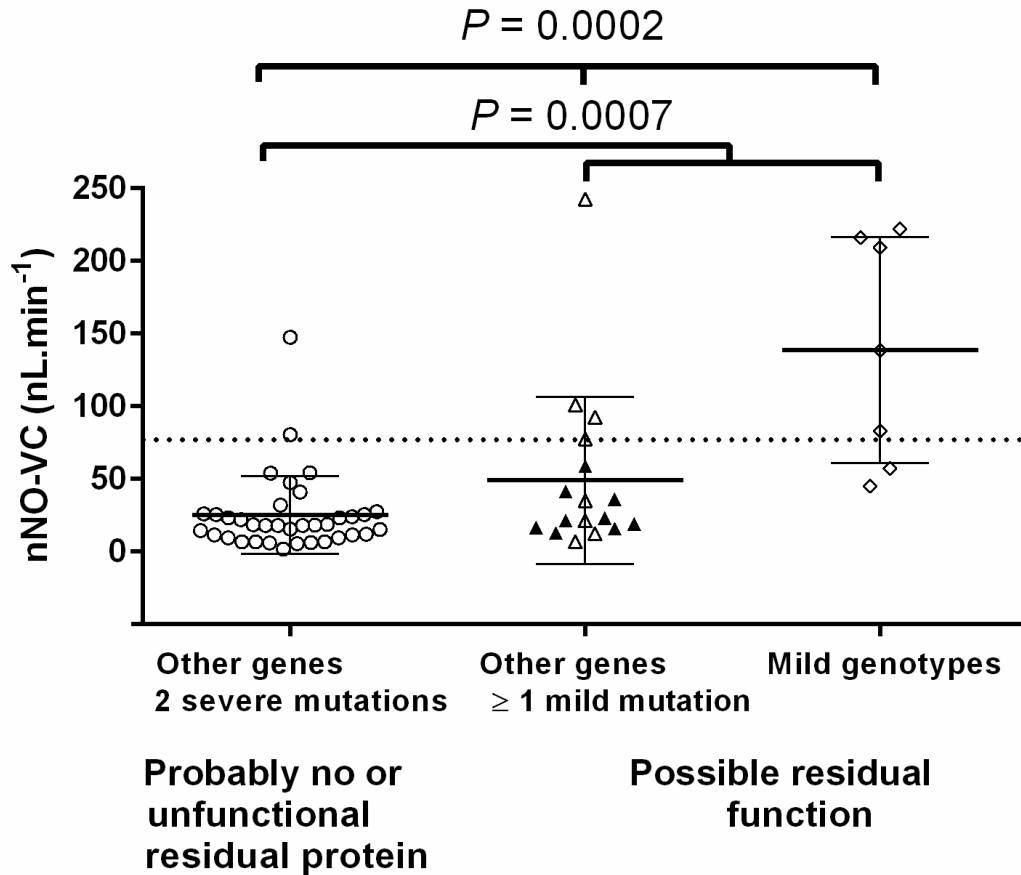


Dotted line is the discriminant threshold(77 nL.min⁻¹).

ODA+IDA: absence of ODA and IDA; ODA: Outer Dynein Arm defect; IDA/MTD: Inner Dynein Arm defect with microtubular disorganisation; CC: Central Complex defect; normal TEM: normal ultrastructure.

Circles are values in children with ODA+IDA, pointing up triangles are values in children with ODA, diamonds are values in children with IDA/MTD, pointing down triangles are values in children with CC, and squares are values in children with nEM.

Figure 3 – Distribution of nasal Nitric Oxide (nNO) measured with velum closed according to genes and mutations in 60 children

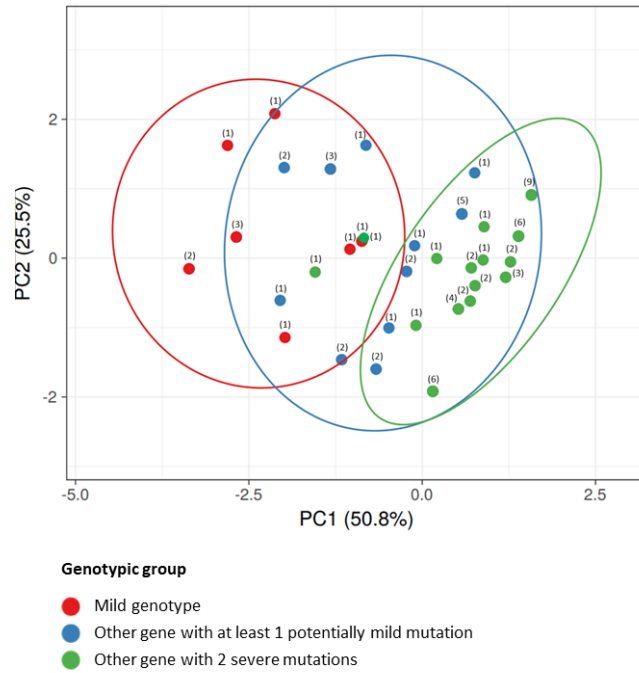


Dotted line is the discriminant threshold (77 nL.min⁻¹).

Circles are values in children with two severe mutations in other-than-mild genes, triangles are values in children with one (open triangles) or two (closed triangles) potentially mild mutations in other-than-mild genes, diamonds are values in children with mild genotypes (*RSPH1* or *CCDC103* p.His154Pro mutations).

There was also a significantly difference between the “Other genes 2 severe mutations” group and the two other genotype groups (versus “Other genes with ≥ 1 potentially mild mutation”; $P = 0.048$, versus “Mild genotypes”; $P < 0.0001$).

Figure 4 – Clustering of the 73 PCD patients according to their genotype, nNO levels, ciliary motility, and ciliary ultrastructure (principal component analysis with ClustVis tool, singular value decomposition).



Input data include: assumed severity of the genotype, nNO level, ciliary motility and TEM defect (each scored as detailed in Material and Methods section). X and Y axes respectively show principal component 1 (PC1) and principal component 2 (PC2) that explain 50.8% and 25.5% of the total variance. Each point represents a patient; points with identical input are superposed (number of patients are mentioned in brackets above each point). Prediction ellipses indicate that a new observation from the same group will fall inside the ellipse with a 95% probability.

PCD: primary ciliary dyskinesia