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Early View

Original article

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Whole Exome Sequencing Accuracy in the Diagnosis of Primary Ciliary Dyskinesia

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Take home message:

Untargeted whole exome sequencing in subjects with clinical symptoms highly suggestive of PCD has an excellent diagnostic accuracy and as prices drop may be the genetic test of choice for confirming PCD or establishing an alternative diagnosis.

ABSTRACT

The diagnosis of Primary Ciliary Dyskinesia (PCD) relies on clinical features and sophisticated studies. The detection of bi-allelic disease-causing variants confirms the diagnosis. However, a standardized genetic panel is not widely available and new disease-causing genes are continuously identified.

To assess the accuracy of untargeted whole-exome sequencing (WES) as a diagnostic tool for PCD, patients with symptoms highly suggestive of PCD were consecutively included. Patients underwent measurement of nasal nitric oxide (nNO) levels, ciliary transmission electron microscopy analysis (TEM) and WES. A confirmed PCD diagnosis in symptomatic patients was defined as a recognized ciliary ultrastructural defect on TEM and/or two pathogenic variants in a known PCD-causing gene.

Forty-eight patients (46% male) were enrolled, with a median age of 10.0 years (range 1.0 - 37 years). In 36 patients (75%) a diagnosis of PCD was confirmed, of which 14 (39%) patients had normal TEM. A standalone untargeted WES had a diagnostic yield of 94%, identifying bi-allelic variants in eleven known PCD causing genes in 34 subjects. A nNO<77nl/min was nonspecific when including patients younger than 5 years (area under the ROC curve (AUC) 0.75 (95%CI, 0.60-0.90)). Consecutive WES considerably improved the diagnostic accuracy of nNO in young children (AUC 0.97 (95%CI, 0.93-1). Finally, WES established an alternative diagnosis in four patients.

In patients with clinically suspected PCD and low nNO levels, WES is a simple, beneficial and accurate next step to confirm the diagnosis of PCD or suggest an alternative diagnosis, especially in preschool-aged children in whom nNO is less specific.

INTRODUCTION

Primary ciliary dyskinesia (PCD) is a rare, predominantly autosomal recessive genetic disorder, caused by defects in ciliary structure and function. Mutations in PCD associated genes can lead to impaired ciliary motility, or paucity of cilia, resulting in impaired mucociliary clearance and progressive lung disease[1, 2]. Historically, the gold standard for the diagnosis of PCD had been evidence of an ultrastructural ciliary defect as seen on transmission electron microscopy (TEM)[3]. However, due to high false negative rates, alternative diagnostic tests were evaluated in the last decade[4-9]. Guidelines for the diagnosis of PCD have recently been published by the American Thoracic Society (ATS)[10] and the European Respiratory Society (ERS)[11]. These include the use of diagnostic modalities such as scrape or brush biopsies for ciliary TEM analysis, high-speed video microscopy (HSVM) with ciliary beat frequency, and the use of nasal nitric oxide (nNO) measurements. These modalities require expertise, and each has its limitations[12-15]. Recent advances and availability of genetic diagnostic tools, including the availability of commercial gene panels, can help confirm the diagnosis of PCD, which allows early diagnosis, family planning, genotype-phenotype correlation and possibly individualized treatment in the future. Indeed, the ATS recommends the use of an extended genetic panel for PCD diagnosis in patients fulfilling clinical criteria, with low nNO, superseding the use of TEM[16]. This recommendation is based on expert opinion, as the different panels have not been evaluated in prospective studies. Untargeted whole-exome sequencing (WES) is an efficient and increasingly economical genetic analysis method which is not limited to a panel of targeted pathogenic variants or known disease-related genes. It is increasingly available to most medical centers and can provide extensive genetic information which may help in confirming PCD diagnosis or provide alternate diagnoses[17]. The aim of this current study is to assess

the diagnostic yield of WES, incorporated into the evaluation of patients with high clinical suspicion of PCD, in a real-life clinical setting.

METHODS

The study was approved by the Institutional Review Board, and consent was obtained from patients or their legal guardians. A more detailed explanation of the methods is provided in the online supplement.

Patients

Individuals with a chronic sinopulmonary disease evaluated between 2012 and 2019 in whom cystic fibrosis and immune deficiency were ruled out, were prospectively recruited for this study. Patients were included if they consented to perform WES and had a very high clinical suspicion for PCD: Term born with chronic sinopulmonary symptoms since early childhood and one or more of the following criteria; (1) unexplained bronchiectasis, (2) a condition associated with PCD (situs inversus totalis or any heterotaxic syndrome), (3) a history of otherwise unexplained neonatal respiratory distress. Using these clinical criteria, we expected a pre-evaluation sensitivity of above 70% for a PCD diagnosis in our cohort [5, 18].

Data and sample collection

All subjects underwent detailed clinical assessment, nNO measurements, and TEM from nasal brush biopsy, if not previously performed.

In cooperative patients, nNO sampling was performed with palate closure maneuver[19]. In non-cooperative patients, nNO was measured during tidal breathing[20]. Both nNO production values of 30 $nL \cdot min^{-1}$ (90 ppb for uncooperating subjects) and 77 $nL \cdot min^{-1}$ (233 ppb for uncooperating subjects) have been recommended as cut-off values for evaluation of PCD and were thus both assessed in this study [15, 21].

A nasal brush biopsy was performed under local anesthesia or during a clinically indicated bronchoscopy and was immediately fixed in glutaraldehyde for TEM analysis. TEM was analyzed by a pathologist experienced in the diagnosis of PCD. Ciliary ultrastructure was

described as either “hallmark” abnormal, abnormal but suspected secondary ciliary defect[22], inadequate, inconclusive, or normal[10, 12].

Whole Exome Analysis

Following DNA extraction from whole blood, exonic sequences were enriched with the SureSelect Human All Exon 50 Mb V5 Kit (Agilent Technologies, Santa Clara, California, USA). Sequences were generated on a HiSeq2500 (Illumina, San Diego, California, USA) as 125-bp paired-end runs. Read alignment and variant calling were performed with DNAnexus (Palo Alto, California, USA) with the human genome assembly hg19 (GRCh37) as reference. Data analysis was performed using an in-house bioinformatics pipeline, exomes were covered at above 50X depth.

Definitions

A positive diagnosis[12], was defined as 1) the presence of a typical clinical phenotype plus a recognized ciliary ultrastructural defect or 2) a typical clinical phenotype and the presence of two pathogenic variants in a known gene causing PCD.

A negative diagnosis was reported in subjects if 1) an alternative diagnosis was established or 2) all laboratory diagnostic studies were not consistent with PCD.

An unresolved diagnosis was reported in patients with a typical clinical phenotype if nNO levels were low and ciliary ultrastructure was normal or inconclusive and no pathogenic variants in a ciliary gene were found.

Statistical Analysis

Proportional differences were assessed using the Chi-squared and Fisher’s exact tests for nominal variables and T-test and the Mann-Whitney for continuous variables. The diagnostic accuracy of each laboratory test was determined based on the final diagnosis. Patients with an unresolved diagnosis were not excluded but considered as a negative diagnosis for PCD.

Sensitivity, specificity, positive and negative predictive values and area under the receiver operating characteristic (ROC) curve were calculated for each diagnostic evaluation as well as a combined evaluation of low nNO levels and WES. All analyses were performed using STATA 15.1 (Stata Corp TX, USA).

RESULTS

Study Population

Forty-eight subjects were evaluated in the study (46% male, median age of 10.0 years (range: 1-37 years). Of the 48 subjects included in the study, 36 (75%) were diagnosed with definite PCD, in four (14%) patients the diagnosis was inconclusive and in eight (22%) patients PCD was ruled out (Figure 1). Additional patient characteristics are presented in Table 1.

Patients diagnosed with PCD

Of the 36 individuals diagnosed with PCD, in 34 WES identified bi-allelic variants in eleven previously reported motile cilia genes (Tables 2 and E1) (94% diagnostic yield). The specific variants identified in these motile cilia genes are presented in Table E1. In one adult patient with a classical PCD phenotype (bronchiectasis, low nNO levels and missing dynein arms on TEM) WES identified two likely pathogenic variants in an axonemal cilia gene (*DNAH10*), predicted to be PCD causing [23] but not previously reported as disease-causing. As per our definitions for the purpose of assessment of the diagnostic accuracy of WES (and due to lack of functional studies), this was considered false negative. WES did not identify any pathogenic variants in one patient with a classic PCD phenotype (chronic purulent otitis media, bronchiectasis, low nasal NO and positive TEM with absent dynein arms).

Genotype-phenotype

In terms of genotype-phenotype association, patients with variants in *LLRC6* and *LLRC50* genes had a high prevalence of situs abnormalities, history of neonatal respiratory distress

and were associated with both outer and inner dynein arm defects on TEM. Patients with variants in *HYDIN*, *RSPH4A*, and *RSPH9* genes had normal TEM and no situs abnormalities. Other patients' characteristics and associated genetic variants are presented in Table 2.

Patients with an alternative diagnosis

In four subjects, WES established an alternative diagnosis. One subject was diagnosed with immune deficiency due to an autosomal dominant *STAT1* gain of function variant (OMIM #614162). TEM, in this patient, initially showed dynein arm and central pair defects, but a repeated nasal brush biopsy demonstrated normal ciliary structure (possibly reflecting a secondary defect observed by the initial TEM). Two additional individuals, from one large consanguineous family, were diagnosed with plasminogen deficiency (OMIM #217090, *PLG* gene) confirmed by repeated low plasma plasminogen levels. These individuals had low nNO levels (under 30 $nL \cdot min^{-1}$) on multiple occasions and recurrent inadequate samples for TEM evaluation. In a fourth individual (suffering from situs inversus and mild respiratory symptoms), WES revealed two pathogenic variants in *WDR16*, previously described to cause laterality disorders without evidence of ciliary dysfunction[24].

Patients with an inconclusive diagnosis

In four subjects (8.3%) (mean age 6.5 years (range: 4-10 years)), a final diagnosis could not be reached. These four subjects all had a nNO level below 77 $nL \cdot min^{-1}$ and normal ciliary ultrastructure on TEM. WES did not identify a genetic cause for their symptoms. Thus, for the purpose of the diagnostic evaluation of WES results in these subjects were considered true negative.

However, according to current ATS criteria[10] these subjects might be diagnosed as patients with PCD. Thus, an additional evaluation was performed considering WES results in these patients as false negative (see below).

Accuracy of the Diagnostic Evaluation

As a standalone test, WES had the highest accuracy with an AUC=0.97 (95% CI, 0.93-1), followed by nNO < 30 $nL \cdot min^{-1}$ (90ppb) with AUC=0.84 (95% CI, 0.71-0.98). The sensitivity and specificity of WES for the diagnosis of PCD were 94.4% (95% CI, 81.3-99.3), 100% (95% CI, 73.5-100), respectively (Table 3).

In view of current ATS criteria suggesting a diagnosis of PCD in symptomatic patients and low nNO levels, with no need of auxiliary testing, the diagnostic accuracy of WES according to current ATS criteria was also evaluated. This showed a reduced diagnostic accuracy with an AUC=0.91 (95% CI, 0.85-0.98) due to a reduced sensitivity of 83.3% (95% CI, 67.2-93.6).

WES was also assessed as an auxiliary test following nNO. The added value of WES was particularly evident in young subjects (≤ 5yr), in whom nNO <77 $nL \cdot min^{-1}$ (233ppb) alone had an AUC of 0.67 (95%CI 0.46-0.87), while the combination of nNO<77 $nL \cdot min^{-1}$ and consecutive WES yielded an AUC of 0.97 (95%CI 0.93-1.00) (Table 4).

Excluding patients with unresolved diagnosis from the analysis (as they may be regarded as true negative or false negative[10]) considerably improved the diagnostic abilities of nNO, especially in individuals above 5 years of age (Table 4).

Cost comparison WES vs commercially available genetic panel for PCD

The current cost of WES is estimated at 1000 USD, with clinical WES prices ranging between $500-5000[25], depending on insurance participation.

The cost of a commercial genetic panel for PCD is estimated at 300 USD (personal communication). The use of WES in our study group yielded a genetic diagnosis in 39 subjects (PCD and non-PCD). Theoretically, a commonly used specific commercial genetic PCD panel[26] for these 48 subjects would have cost 3.3 times less but resulted in genetic diagnosis in 31 subjects (1.25 times less). Naturally, custom-made panels are also available which may increase the diagnostic yield as well as the cost.

DISCUSSION

In this study, we evaluated, in a real-life setting, the yield of untargeted WES as a diagnostic tool for PCD. Our findings demonstrate that untargeted WES in subjects with clinical symptoms highly suggestive of PCD has excellent sensitivity and specificity for diagnosis. Furthermore, untargeted WES allows identification of new PCD causing genes, novel variants in known PCD genes, and diagnosis of alternative genetic disorders. We propose that WES should be the genetic diagnostic tool of choice in subjects with high clinical suspicion of PCD and low nNO levels and or abnormal/equivocal results using other available diagnostic modalities.

PCD is a rare condition with a prevalence of 1:10,000-20,000, that has nonspecific signs and symptoms shared with many other conditions, and thus requires evaluation in centers with expertise in its diagnosis and treatment. The diagnosis is complicated by the fact that the tests employed in the diagnostic process of PCD are cumbersome, expensive, require expertise not universally available, and there is no single test that can be considered a gold standard for diagnosis. Bearing these hurdles in mind, our study validates current expert opinion-based guidelines[21, 27], incorporating a combination of clinical history, nNO measurement and WES as a highly accurate pathway in establishing a diagnosis of PCD.

Though the genetic approach to PCD diagnosis has developed in recent years, the evidence for its use, as a diagnostic tool is still scarce. Therefore, most guidelines, both American and European recommend it as an auxiliary tool to confirm a PCD diagnosis[10, 28, 29]. Over 40 PCD causing genes have been identified to date, but in only 65%-75% of the patients, a genetic diagnosis is reached using traditional tools[18, 30]. The genotype-phenotype association has been described[31, 32], providing a clinical rationale to achieve a genetic diagnosis, especially with current progression towards precision medicine.

The use of whole-exome sequencing as part of the routine diagnostic pathway in patients with suspected PCD has not been reported previously. Several studies evaluated the ability of targeted and untargeted WES to detect pathogenic variants in patients previously diagnosed with PCD, with an overall yield of 50% to 76%[30, 33-35]. Our prospective evaluation, using an untargeted WES approach in patients with clinical symptoms highly suggestive of PCD, provided a genetic diagnosis in 34 out of 36 individuals with a confirmed PCD diagnosis (94% yield).

In a heterogeneous disease, such as PCD with the constant discovery of new causative genes, the use of WES is practical. Furthermore, WES is becoming increasingly affordable, it does not require expertise present only in PCD centers and the data generated may be repeatedly accessed as genetic databases expand, further improving its future diagnostic yield. Indeed, in three individuals in current study, only a repeated assessment of undiagnostic WES (as genetic databases improved in power and precision) allowed to establish a genetic diagnosis.

At least 8 (14%) subjects in this study benefited from the unbiased WES approach, as opposed to a PCD-targeted genetic panel testing: in 4 individuals WES provided an alternative diagnosis, and in 4 other subjects, pathogenic variants in PCD-causing genes not included in a commercially available panel were identified[26]. Still, when evaluating the cost of WES compared to the current PCD-targeted genetic panel, WES was 3 times more expensive while genetically diagnosing an additional 25% patients. Therefore, our data suggest that until prices of WES are reduced, it would be cost-effective to perform WES only after receiving non-diagnostic PCD-targeted genetic panel results. Importantly, the financial burden of misdiagnosing patients should also be considered, as this often leads to unnecessary diagnostic tests and treatments, as was recently reported[36].

The nNO is consistently low in cooperative older patients with PCD (>5 years old)[6], with a sensitivity and specify above 95% for the diagnosis of PCD, outperforming TEM or TEM

with focused genetic testing[15]. Even in infants younger than 1 year of age, tidal breathing nNO has been shown to have a diagnostic value[37] but is not routinely recommended for use under 5 years of age due to low specificity. In line with the aforementioned studies, we have found excellent predictive values using 77 or 30 $nL \cdot min^{-1}$ cut-offs for PCD diagnosis. nNO in our cohort was both feasible and useful in supporting the clinical diagnosis of PCD. Nonetheless, caution should be exercised using nNO as the final standalone test to diagnose PCD, due to possible false-positive results as exemplified in recent reports[38, 39] as well as in the current study. This is particularly true when evaluating children younger than 5 years in whom low nNO values are non-specific. In the young patients in our cohort, the addition of WES increased the diagnostic yield significantly, as compared to standalone nNO measurements. An early definitive genetic diagnosis allows disease-directed treatment at a specialized clinic which may potentially improve long term outcomes[40]. Furthermore, it provides the family with a genetic cause for their child's symptoms, the option of family planning, and in the future may direct personalized treatment. Finally, it should be noted that our cohort did not include patients with genotypes associated with higher levels of nNO[41], potentially lowering the false-negative predictive value of high nNO in other patient populations, and thus additionally adding to the usefulness of WES.

Four individuals with symptoms suggestive of PCD and low nNO levels underwent WES without a definitive genetic diagnosis. This may be the result of mutations in intronic regions, not covered by WES or due to novel unidentified PCD causing genes. Moreover, mutations may have been missed due to the calling algorithms used for analysis. Future re-analysis using improved algorithms and use of exome trios may allow the identification of additional disease-causing mutations[42].

Some limitations of this study should be noted. First, in view of our strict inclusion criteria, the population in which the WES was assessed had a very high pre-evaluation likelihood for

a PCD diagnosis[18]. Thus, our results are relevant for a specific population of patients with high clinical suspicion for PCD or patients with suggestive symptoms and low nNO levels and may not reflect patients with mild symptoms undergoing routine evaluation for chronic respiratory symptoms. Secondly, as there is no single gold-standard test for diagnosis of PCD, in the current study (similar to other studies on this rare disorder) WES results were used as part of the diagnostic pathway. However, only genetic mutations which would have been reported by appropriate genetic panels, were regarded as true positive. This difficulty was previously discussed by the 2016 ERS diagnostic guidelines concluding that since no single test rules out PCD, a diagnostic modality can be evaluated even though it is used in the diagnostic pathway by comparing the accuracy of the test with the patient's final outcome[12]. Thirdly, the specific population in which the diagnostic modality was assessed. The Hadassah Medical Center, is a regional referral center for chronic pulmonary conditions specializing in PCD diagnosis. The population basis is 1.5 million residents of the larger Jerusalem area and its surroundings. The Arab Muslims, who have a high degree of consanguinity, comprise ~25% of this population but represent 73% of our cohort. Thus, the predictive value of our approach should be tested in other populations.

In conclusion, we have shown that in patients with a highly suggestive clinical history, WES has a very high accuracy and is beneficial for PCD diagnosis, especially in young children. Therefore, as prices of WES drop and expertise is increasing, consecutive testing of WES following low nNO levels or in patients with highly suggestive symptoms, provides an objective, practical and definitive tool for diagnosing patients with PCD.

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Table 1. Characteristics of patients evaluated for Primary Ciliary Dyskinesia

	All patients	**Positive**	**Negative**	**Inconclusive**
Subjects	48	36	8	4
Median age at assessment, yr (range)	10.0 (1-37)	12.1 (1.0-37)	9.6 (1-23)	6.5 (4-10)
0 to 5 yr, n (%)	13 (27)	7 (21)	4 (50)	2 (50)
5 to 18 yr, n (%)	27 (56)	22 (61)	3 (38)	2 (50)
Above 18 yr, n (%)	8 (17)	7 (21)	1 (13)	0 (-)
Male, n (%)	21 (44)	14 (39)	5 (63)	2 (50)
Consanguinity, n (%)	34 (71)	32 (76)	2 (50)	4 (100)
Ethnicity				
Jewish, n (%)	13 (27)	7 (19)	4 (50)	2 (50)
Arabic, n (%)	35 (73)	29 (81)	4 (50)	2 (50)
Other, n (%)	-	-	-	-
Bronchiectasis, n (%)	46 (96)	34 (94)	7 (88)	4 (100)
Associated conditions (SA, RP), n (%)	11 (23)	10 (28)	1 (13)	0 (0)
Neonatal Respiratory Distress, n (%)	15 (31)	13 (36)	0 (-)	2 (50)
Diagnostic assessment performed				
nNO measurement, n (%)	48 (100)	36 (100)	8 (100)	4 (100)
TEM for ciliary structure, n (%)	41 (85)	31 (86)	6 (75)	4 (100)

SA - Situs abnormalities; RP - Retinitis Pigmentosa; nNO - nasal nitric oxide; TEM - Transmission electron microscopy;

Table 2. Primary ciliary dyskinesia causing genes in our cohort and the related prevalence of situs abnormalities (S.A.), neonatal distress and associated findings by transmission electron microscopy, nNO measurements.

Gene (n=34)	**Locus**	**Situs abnormalities**	**Neonatal Respiratory Distress**	**TEM**	**nNO (ppb)** mean (range)
DNAAF3 (n=2)	19q13	n=0	n=1	ODA+IDA	46 (32-60)
DNAH11 (n=9)	7p15–21	n=1	n=3	normal	41.8 (10-79)
DNAI1 (n=4)	9p21-p13	n=1	n=1	Normal/short ODA	34.5 (20-70)
DNAI2 (n=3)	17q25.1	n=1	n=2	Normal/orientation/ODA+ IDA	41.3 (30-47)
DYX1C1 (DNAAF4) (n=1)	15q21	n=1	n=0	Short ODA+IDA	55
HYDIN (n=3)	16q22	n=0	n=1	Normal	59.6 (19-90)
LRRC50 (DNAAF1) (n=2)	16q24	n=2	n=2	ODA + IDA	65
LRRC6 (n=6)	8q24	n=4	n=2	ODA + IDA	81.9 (12-328)
MCIDAS (n=2)	5q11	n=0	n=0	Absence of ciliary cells	57 (54-60)
RSPH4A (n=1)	6q22	n=0	n=0	normal	38
RSPH9 (n=1)	6p21	n=0	n=1	normal	48

nNO - nasal nitric oxide; TEM - Transmission electron microscopy; ODA – outer dynein arm; IDA – inner dynein arm

Table 3. Diagnostic accuracy of nasal nitric oxide (nNO), transmission electron microscopy (TEM) and genetic evaluation for diagnosis of Primary Ciliary Dyskinesia.

	nNO < 77 $nL \cdot min^{-1}$	nNO < 30 $nL \cdot min^{-1}$	TEM	WES
All Subjects, including unresolved diagnosis (n=57)	n=48	n=48	n=41	n=48
Sensitivity (95% CI)	100 (89.1 - 100)	93.8 (79.2-99.2)	62.2 (44.8-77.5)	94.4 (81.3-99.3)
Specificity (95% CI)	50.0 (21.1 -78.9)	75.0 (42.8-94.5)	85.7 (57.2-98.2)	100 (73.5 - 100)
ROC area (95% CI)	0.75 (0.60 - 0.90)	0.84 (0.71-0.98)	0.74 (0.62-0.86)	0.97 (0.93 – 1.0)

nNO - nasal nitric oxide; TEM - Transmission electron microscopy; WES – untargeted whole exome sequencing; ROC area – area under the receiver operating curve.

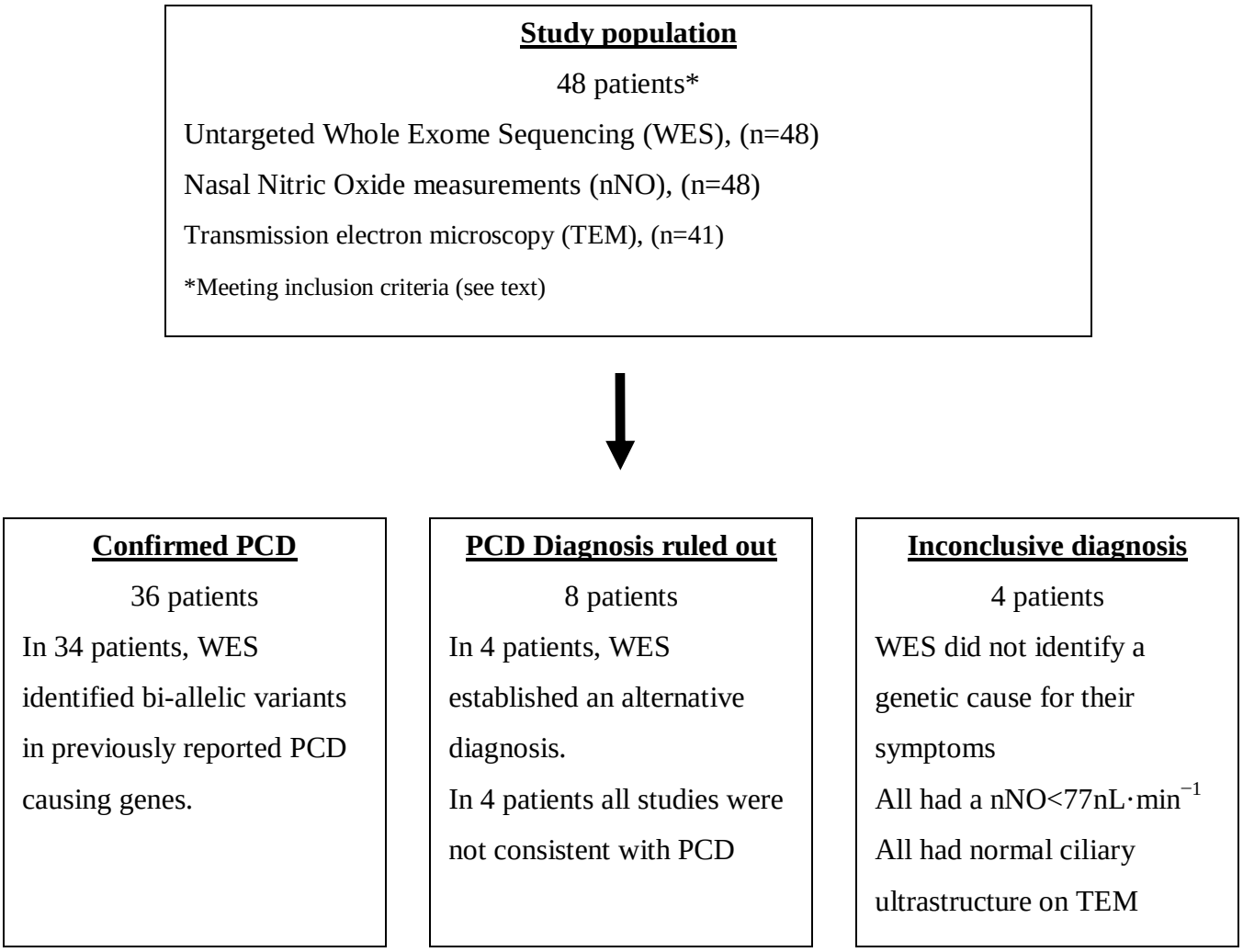
Table 4. Diagnostic accuracy of nasal nitric oxide (nNO)

	nNO < 77 nL·min^{-1}			**nNO < 77 nL·min^{-1} and WES**
All Subjects, including unresolved diagnosis (n=48)	≤ 5 yr	> 5 yr	All	All
Sensitivity (95% CI)	100 (47.8-100)	100 (87.2-100)	100 (89.1 - 100)	93.8 (79.2-99.2)
Specificity (95% CI)	33.3 (4.3-77.7)	66.7 (22.3-95.7)	50.0 (21.1 -78.9)	100 (73.5-100)
ROC area (95% CI)	0.67 (0.46-0.87)	0.83 (0.63-1.00)	0.75 (0.60 - 0.90)	0.97 (0.93-1.0)
Subjects with unresolved diagnosis excluded (n=40)	≤ 5 yr	> 5 yr	All	All
Sensitivity (95% CI)	100 (47.8-100)	100 (87.2-100)	100 (89.1-100)	93.8 (79.2-99.2)
Specificity (95% CI)	50.0 (6.8-93.2)	100 (39.8-100)	75 (34.9-96.8)	100 (63.1-100)
ROC area (95% CI)	0.75 (0.47-1.0)	1.0	0.88 (0.72-1.0)	0.97 (0.93-1.0)

nNO - nasal nitric oxide; WES – untargeted whole exome sequencing

Figure captions

Figure 1. Study population and the results of whole exome sequencing (WES) analysis.


Study population
48 patients*
Untargeted Whole Exome Sequencing (WES), (n=48)
Nasal Nitric Oxide measurements (nNO), (n=48)
Transmission electron microscopy (TEM), (n=41)
*Meeting inclusion criteria (see text)
Confirmed PCD
36 patients
In 34 patients, WES identified bi-allelic variants in previously reported PCD causing genes.
PCD Diagnosis ruled out
8 patients
In 4 patients, WES established an alternative diagnosis.
In 4 patients all studies were not consistent with PCD
Inconclusive diagnosis
4 patients
WES did not identify a genetic cause for their symptoms
All had a nNO<77nL·min^{-1}
All had normal ciliary ultrastructure on TEM

Whole Exome Sequencing Accuracy in the Diagnosis of Primary Ciliary Dyskinesia

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ONLINE DATA SUPPLEMENT

METHODS

The study was carried out in a tertiary referral university medical center. The methodology of the study was approved by the Institutional Review Board, and consent was obtained from patients or their legal guardians.

Patients

Individuals with a chronic sinopulmonary disease evaluated between 2012 and 2019 in whom cystic fibrosis and immune deficiency were ruled out (normal laboratory evaluation including; total blood count, immunoglobulin and immunoglobulin subclasses levels, serologic response to previously administered vaccines, leukocyte immunophenotyping, complement test and in selected cases tests as appropriate, i.e nitroblue tetrazolium test for suspected chronic granulomatous disease), were prospectively recruited for this study. Patients were included if they consented to perform WES and had a very high clinical suspicion for PCD: Term born with chronic sinopulmonary symptoms since early childhood and one or more of the following criteria; (1) unexplained bronchiectasis, (2) a condition associated with PCD (situs inversus totalis or any heterotaxic syndrome), (3) a history of otherwise unexplained neonatal respiratory

distress. Using these clinical criteria, we expected a pre-evaluation sensitivity of above 70% for a PCD diagnosis in our cohort[1, 2].

Data and sample collection

A chart review for missing information was conducted, including previously performed diagnostic testing for PCD. Patients were offered to complete diagnostic evaluation and repeat tests with equivocal results if previously performed. All subjects underwent detailed clinical assessment, nNO measurements and TEM from nasal brush biopsy.

The nNO was measured using a NO Analyzer CLD 88SP, Fa. (Eco Medics, Duernten, Switzerland). In cooperative patients, nNO sampling was performed with palate closure maneuver according to ATS/ERS guidelines [3]. In non-cooperative patients, mainly preschool children, nNO was measured during tidal breathing [4]. In all patients in whom the nNO measurement was low, at least two repeated nNO measurements were performed during separate clinical visits. The highest recorded nNO level was used in the final analysis. Due to the inclusion of nNO measurements in preschool individuals, measured during tidal breathing, we report nNO concentration (parts per billion (ppb)) in addition to nNO production (nl/min). For cooperative patients nNO production (nl/min) can be calculated by multiplying the nNO concentration (PPB) by the sampling flow rate (0.33 $L \cdot min^{-1}$for the CLD device) [5]. A nNO production values of 30 $nL \cdot min^{-1}$ (90 ppb for uncooperating subjects) and 77 $nL \cdot min^{-1}$ (233 ppb for uncooperating subjects) have been both recommended as cut-off values for evaluation of PCD, the former with increased specificity and latter with increased sensitivity, and were both assessed.

A nasal brush biopsy was performed under local anesthesia or during a clinically indicated bronchoscopy and was immediately fixed in glutaraldehyde for TEM analysis. TEM was analyzed by a pathologist experienced in the diagnosis of PCD. Ciliary ultrastructure was described as either "hallmark" abnormal, abnormal but suspected secondary ciliary defect[6], inadequate, inconclusive (no specific hallmark abnormality or a discordance between results from different time points), or normal. Repeat biopsies were offered to patients with suspected secondary defects and inadequate or inconclusive results. As cell culture was not available, suspected secondary defects and inadequate or inconclusive results are reported as such.

Whole Exome Analysis

Following DNA extraction from whole blood, exonic sequences were enriched with the SureSelect Human All Exon 50 Mb V5 Kit (Agilent Technologies, Santa Clara, California, USA). Sequences were generated on a HiSeq2500 (Illumina, San Diego, California, USA) as 125-bp paired-end runs. Read alignment and variant calling were performed with DNAnexus (Palo Alto, California, USA) with the human genome assembly hg19 (GRCh37) as reference. Data analysis was performed using an in-house bioinformatics pipeline.

Definitions

A positive diagnosis, in accordance with current ERS guidelines[7], was defined as 1) the presence of a typical clinical phenotype (as listed in the inclusion criteria) plus a recognized ciliary ultrastructural defect with or without abnormal low nNO levels, or 2) a

typical clinical phenotype and the presence of two pathogenic variants in a known gene causing PCD.

A negative diagnosis was reported in patients with typical clinical phenotype if 1) an alternative diagnosis was established or 2) all laboratory diagnostic studies were not consistent with PCD (high nNO levels, normal ciliary ultrastructure on TEM and no pathogenic variants in a recognized ciliary gene).

An unresolved diagnosis was reported in patients with a typical clinical phenotype if nNO levels were low and ciliary ultrastructure was normal or inconclusive and no pathogenic variants in a ciliary gene were found.

Statistical Analysis

Demographic, clinical, and laboratory variables were summarized by standard descriptive statistics as means and standard deviations for continuous variables and percentages for nominal variables. Proportional differences were assessed using the Chi-squared and Fisher's exact tests for nominal variables and T-test and the Mann-Whitney for continuous variables. Two-sided P values<0.05 were considered to be statistically significant. The diagnostic accuracy of each laboratory test was determined based on the final diagnosis. Patients with an unresolved diagnosis were not excluded but considered as a negative diagnosis for PCD.

Sensitivity, specificity, positive and negative predictive values and area under the receiver operating characteristic (ROC) curve were calculated for each diagnostic evaluation as well as a combined evaluation of low nNO levels and WES. All analyses

were performed using SPSS 25 (IBM Corp, NY, USA) and STATA 15.1 (Stata Corp TX, USA).

Table E1: Variants in ciliary genes identified by WES in PCD patients

GENE	POSITION	TRANSCRIPT	HGVSc	HGVSp	dbSNP	ZYGOSITY	EXPECTED SIGNIFICANCE	REF.
DNAH11	7:21847599	NM_001277115.2	c.10264G>A	p.Gly3422Arg	rs764509824	Homozygous	VOUS	
DNAH11	7:21940758	NM_001277115.2	c.13436_13437insCTGTG	p.Val4480CysfsTer8		Homozygous	expected pathogenic (LOF)	
DNAH11	7:21940756	NM_001277115.2	c.13457_13461dup	p.Tyr4488LeufsTer7		Homozygous	expected pathogenic (LOF)	
DNAH11	7: 21765427	NM_001277115.2	c.7267-2A>T			Homozygous	pathogenic (LOF)	
DNAH11	7:21932181	NM_001277115.2	c.12667G>T	p.Glu4223Ter		Homozygous	pathogenic (LOF)	
DNAI1	9:34517334	NM_012144.4	c.1871delC	p.Pro624LeufsTer66	rs1168493593	Homozygous	expected Pathogenic	
DNAI2	17:72287221	NM_023036.6	c.674delA	p.Asn225ThrfsTer17		Homozygous	expected pathogenic (LOF)	
DNAI2	17:72305484	NM_023036.6	c.1304G>A	p.Trp435Ter	rs752924362	Homozygous	Pathogenic	[8]
DNAI2	17:72281177	NM_023036.6	c.184-2A>T			Compound heterozygous	expected pathogenic (LOF)	
DNAI2	17: 72306303	NM_023036.6	c.1494+1G>A			Compound heterozygous	expected pathogenic (LOF)	
DNAAF3	19:55677272	NM_001256714.1	c.323T>C	p.Leu108Pro	rs387907151	Homozygous	pathogenic	[9]
DYX1C1	15: 55783336	NM_130810.4	c.384_390delCGCACTA	pTyr128del		Homozygous	expected pathogenic (LOF)	
HYDIN	16:71065734	NM_001270974.2	c.2616_2617insTGGCACTGAC	p.Leu873TrpfsTer3		Homozygous	expected pathogenic (LOF)	[10]
HYDIN	16:70989305	NM_001270974.2	c.6289C>T	p.Gln2097Ter	rs774501536	Homozygous	expected pathogenic	
LRRC6	8:133645203	NM_012472.6	c.436G>C	p.Asp146His	rs200321595	Homozygous	pathogenic	[11]
LRRC6	8:133673804	NM_012472.6	c.79_80delTC	p.Ser27ValfsTer13	rs769220870	Homozygous	expected pathogenic (LOF)	
LRRC50	16:84203779	NM_178452.6	c.1349_1350insC	pAsn230Asp	rs397515339	Homozygous	pathogenic	[12]

MCIDAS	5:54516210	NM_001190787.3	c.1142G>A	p.Arg381His	rs797045152	Homozygous	pathogenic	[13]
RSPH4A	6:116949263	NM_001010892.3	c.1393C>T	p.Arg465Ter	rs755782051		pathogenic	[14]
RSPH9	6:43638659	NM_001193341.1	c.801_803GAA	p.Lys268del	rs397515340	Homozygous	pathogenic	[15]

* dbSNP: The Single Nucleotide Polymorphism Database; HGVSc: Human Genome Variation Society nomenclature, coding; HGVSp: Human Genome Variation Society, protein change; LOF: loss-of-function; Ref: Reference; VOUS: variant of unknown significance.

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