Original Article

An international collaborative initiative to screen primary ciliary dyskinesia with high-speed video microscopy and nasal nitric oxide at a tertiary referral centre in South India

Antony T. Benjamin[1,2], Ram Ganesh[1], Kalyanasundaram Muthuswamy[3], Vijayakumar Raju[3], Madhumitha Subramanium[2], Madhumitha K.[2], Claire L. Jackson[4,5], Janice Coles[4,5], Sharada Iyer[6], Jacinta D'souza[6], Jane S Lucas[4,5]

[1]Primary Ciliary Dyskinesia Unit, G. Kuppuswamy Naidu Memorial Hospital, Coimbatore, Tamil Nadu, India, [2]Department of Paediatrics, G. Kuppuswamy Naidu Memorial Hospital, Coimbatore, Tamil Nadu, India, [3]Paediatric Cardiology and Cardiothoracic Surgery, G. Kuppuswamy Naidu Memorial Hospital, Coimbatore, Tamil Nadu, India, [4]Clinical and Experimental Sciences, University of Southampton Faculty of Medicine, Southampton, UK, [5]Primary Ciliary Dyskinesia Centre, University Hospital Southampton NHS Foundation Trust, Southampton, UK, [6]School of Biological Sciences, UM-DAE Center for Excellence in Basic Sciences, Mumbai, Maharashtra, India

ABSTRACT

Background and Objective: To screen patients with suspected primary ciliary dyskinesia (PCD) using high-speed video microscopy analysis (HSVA) and/or nasal nitric oxide (nNO) through an international collaborative initiative between GKNM Hospital, Coimbatore, India, and the PCD Centre, University Hospital Southampton, UK. **Methods:** Children and adults up to 30 years of age with clinical features suggestive of PCD were recruited. HSVA was performed on nasal brushing biopsies for all participants. Nasal NO was measured, when feasible, in patients aged ≥5 years using a portable electrochemical NIOX VERO® device. Online multidisciplinary team meetings involving both centres reviewed and interpreted the results. Patients who tested positive on at least one of the two screening tests were classified as PCD screen-positive. Due to resource constraints, only a subset of screen-positive patients underwent genetic testing. **Results:** Among the 113 patients screened, 68 (60.1%) were PCD screen-positive. Next-generation sequencing was performed in 26 of these 68 patients, and PCD was genetically confirmed in 14 cases. The median age at diagnosis was 8.5 years. **Conclusion:** In resource-limited settings, diagnosis of PCD is often delayed. A combined screening strategy using HSVA and nasal NO testing can help identify likely PCD cases and facilitate early intervention. HSVA and nNO testing were found to be complementary in the screening of PCD. Genetic testing was used in a subset of patients to confirm diagnosis and was conclusive in 54% of tested.

KEY WORDS: High-speed video microscopy, nasal nitric oxide, primary ciliary dyskinesia

Address for correspondence: Dr. Antony T. Benjamin, Primary Ciliary Dyskinesia Unit, G. Kuppuswamy Naidu Memorial Hospital Department of Paediatrics, G. Kuppuswamy Naidu Memorial Hospital, Coimbatore, Tamil Nadu, India.
E-mail: antonyterance@gmail.com

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INTRODUCTION

Primary ciliary dyskinesia (PCD) is a rare genetic disease affecting motile cilia, predominantly inherited as an autosomal recessive disorder. It is a clinically and genetically heterogeneous syndrome involving multiple genes affecting multiple organs, and disease-causing variants occur in more than 56 genes. Global PCD prevalence is estimated to affect 1:7500.[1] The symptoms of PCD overlap with other common conditions causing respiratory illness in children. A European survey reported that 37% of patients had visited more than 40 times before being referred for PCD diagnostic tests.[2]

PCD produces manifestations in all the organs with motile cilia. Patients develop symptoms from the newborn period, manifesting as unexplained respiratory distress, rhino-sinusitis, and persistent wet cough eventually leading to bronchiectasis. Situs abnormalities are present in approximately 50% of children with PCD.[3] Clinical predictive tools, the PrImary CiliAry DyskinesiA Rule (PICADAR),[4] and North American criteria-defined clinical features[5] are used to screen patients for referral to diagnostic facilities. The diagnosis of PCD is challenging as there is no single gold-standard test available for diagnosing PCD. It is diagnosed using a combination of tests that require expensive equipment and expertise.[6] Most centres use a diagnostic algorithm using a different combination of the following five tests: nNO, HSVA, transmission electron microscopy (TEM), immunofluorescence microscopy (IF), and genetic testing. There are European and American consensus guidelines, but it is difficult to standardise protocols globally and with limited resources.[7]

Early diagnosis and intervention can limit disease progression and prevent complications.[8] Being a genetic disease, siblings of children with PCD should also be screened and genetic counselling should be given.

A PCD diagnosis is more difficult in resource-limited settings due to lack of awareness about PCD and a paucity of diagnostic facilities.[9] In 2018, a new PCD diagnostic facility was set up at GKNM Hospital Coimbatore, South India in collaboration with the PCD diagnostic unit, University Hospital, Southampton, UK. As a result, we screened and diagnosed referral patients and shared anonymised clinical, phenotypic, and genetic profiles with the Southampton PCD service to diagnose PCD patients at our centre in South India.[10-12] We are presenting our single centre experience in PCD screening using HSVA and nNO.

MATERIALS AND METHODS

Patients with suspected PCD were identified when they have a combination of two or more of the cardinal features: term neonate with unexplained respiratory distress, early onset persistent wet cough from infancy, early onset persistent nasal discharge from infancy/ear disease, and organ laterality defects. Siblings of patients with PCD and patients with unexplained bronchiectasis were also included in the study. Ethical approval was obtained from G. Kuppuswamy Naidu Memorial Hospital institutional ethical committee on 14 December 2017 (reference number: ECR/209/inst/TN/2013/RR-19), and informed consent was obtained. A proforma was used to collect patient demographic details and clinical features. Nasal brush biopsies for HSVA were done in all patients, while nNO was performed when feasible in patients who were at least 5 years of age and test-competent. The results of HSVA and nNO were available on the same day for interpretation, and when tests were abnormal, treatment was initiated.

Nasal NO was measured in children ≥5 years of age using a portable electrochemical analyzer, NIOX VERO® equipment with a sampling rate of 5mL/s. A paediatric nasal olive was placed into one nostril to sample air, and the patient performed a velum closure manoeuvre[13] by exhaling through the mouth against a resistor. For children who could not perform the expiration against resistance manoeuvre (ER-nNO), nNO measurement was done during tidal breathing through open mouth (TB-nNO).[13] The NIOX VERO® device aspirates air from the nasal cavity for 30 seconds to deliver a measurement. One measurement was made from each nostril, and the highest reading was reported. The cut-off values for ER-nNO and TB-nNO were 356 ppb and 171 ppb, respectively (equating to 107 nL/min and 51 nL/min).[14] Random measurements of ambient NO concentrations revealed levels below 5 ppb, suggesting no significant ambient NO pollution.

A nasal brush biopsy was performed only when the patients were free of upper respiratory infections, to reduce the likelihood of secondary ciliary dyskinesia that may confound HSVA results. Respiratory epithelium was sampled using a cytology brush (3 × 10 mm) on the lateral wall of the inferior nasal turbinate; the brush was immersed into 2 ml of Medium 199 and vigorously agitated to discharge epithelial cell clusters into the media. Approximately 100 µL of cell suspension was transferred onto a glass slide, sealed with a coverslip. The slides were assessed by light microscopy for health and cell type. Healthy epithelial cell clusters were identified as having at least 5 cells per cluster and a viable appearance. Nasal epithelial cell clusters were then viewed using an oil immersion, 100x objective lens, with 10x optical zoom. Ciliary movement was captured using a Promon U750 high-speed video camera at 200 frames per second (fps). The video recordings were reviewed at a slower frame rate of 20 fps using AOS Studio v4 imaging software. A minimum of eight recordings were made per patient sample, which included seven side views (imaging cilia laterally) and at least one top view (imaging cilia vertically). Ciliary beat pattern was described, and ciliary beat frequency was measured in

Hertz. Results of HSVA were classified as 1) normal, 2) abnormal, or 3) indeterminate. Normal ciliary beating had regular and coordinated forward and recovery strokes. Abnormal ciliary beating was categorised as immotile cilia, minimal residual motility, stiff beating, circular beating, and absent cilia.[15] The indeterminate category included poor quality specimens or inadequate samples, which were not included in these data, and repeat patient sampling was needed. Recordings and observations were made at room temperature (approximately 23°C) due to lack of an environmental chamber. All recorded videos were archived on the hospital server. Authors 1 and 2 were trained on HSVA at the University Hospital, Southampton PCD diagnostic centre, UK. Our HSVA recordings and reports were reviewed in cross-centre multidisciplinary meetings with the Southampton PCD team via video conferencing. Patients were categorised as PCD (highly likely) if at least one of the two screening tests were positive (abnormal HSVA, low nNO, as already described).

Blood samples from patients who had PCD (highly likely) by abnormal HSVA and/or nNO outcomes were outsourced to a genomics laboratory for next-generation gDNA sequencing. Selective capture (at least 30 PCD genes) and sequencing of the protein-coding regions was performed. Variants identified in the exonic regions were generally clinically actionable compared to variants of uncertain significance (VUS) or non-coding region variants occasionally seen close to exon regions. The libraries were sequenced to a mean depth of >80-100X using an Illumina sequencing platform. The GATK best practices framework for identifying germline variants in the sample were followed using Sentieon.[16] The sequences obtained were aligned to the human reference genome (GRCh38) using BWA aligner and analysed using Sentieon to remove duplicates, recalibration, and re-align indels.[16,17] A Sentieon haplotype caller was used to identify variants in the sample. Germline variants identified in the sample were annotated using VariMAT pipeline. Gene annotation of the variants was performed using VEP program[18] against the Ensembl release 99 human gene model.[19] In addition to single nucleotide variants (SNVs) and small indels, copy number variants (CNVs) were detected from targeted sequence data using the Exome Depth method.[20] This algorithm detects CNVs by comparing the read depths in the sample of interest with the matched aggregate reference dataset.

Clinically relevant mutations were annotated using published variants in the literature and a set of disease databases – ClinVar, OMIM (updated on 20th Feb 2020), GWAS, HGMD (v2019.4), and SwissVar.[21-25] Common variants were filtered based on allele frequency in 1000Genome Phase 3, gnomAD (v2.1), EVS, dbSNP (v151), 1000 Japanese Genome, and our internal Indian population database.[26-30] The non-synonymous variant effect was calculated using multiple algorithms such as PolyPhen-2, SIFT, MutationTaster2, and LRT. Candidate variant pathogenicity was evaluated as per the criteria of the American College of Medical Genetics and Genomics (ACMG).[31] Silent variations that do not change amino acids in the coding region were not reported.

Statistical analysis

All continuous variables were represented as median, and range based on data distribution, while categorical variables were presented as frequency and percentage. All analyses were carried out using Jamovi version 2.3.21.

RESULTS

113 patients with clinical features suggestive of PCD were recruited [Figure 1]. All 113 patients underwent HSVA, of which 87 (76.9%) had a conclusive result. 26 patients had inconclusive results due to either inadequate or poor sample quality. For patients with conclusive results, HSVA abnormality was seen in 48 (42.4%) patients. The types of HSVA abnormalities were immotile cilia 20 (41.6%), stiff motility 18 (37.5%), minimal residual motility 4 (8.3%), rotatory motility 3 (6.3%), and absent cilia 3 (6.3%). Nasal nitric oxide was performed in 71 patients, ER-nNO in 44 (62%), and TB-nNO in 27 (38%) patients. nNO was found to be low in 57 (80%) patients.

Out of 113 patients, 68 (60.1%) had at least one screening test positive, and these patients were categorised as PCD screen-positive. Among these 68 patients who had PCD (screen-positive), only 37 patients had both screening tests positive [Figure 2]. HSVA was abnormal in 11 patients who could not perform nNO and nNO was low in 20 patients where HSVA was inconclusive.

NGS could be done in only 26 out of 68 PCD (screen-positive) patients who afforded genetic testing, and the samples were outsourced. 14 subjects carried pathogenic/likely pathogenic (LP) biallelic mutations in at least one PCD causal gene, with a genetic detection rate of 53.8% [Table 1]. Genetic analyses revealed biallelic pathogenic variants in 9 PCD genes: *CCNO*, *DNAH5*, *DNAH11*, *HYDIN*, *CCDC39*, *CCDC164*, *DNAAF2*, *DNAH1,* and *ODAD4* [Figure 3]. Two families had two siblings each who shared the same mutation in *CCNO* and *HYDIN* genes. Biallelic VUS accounted for 5 (19.2%) patients. Mono-allelic variants were identified in 5 (19.2%) patients and no mutations in 2 (7.8%) patients.

The median age at the time of diagnosis for these 14 confirmed PCD patients was 8.5 years [Table 2]. Three patients (21.4%) with laterality defects were identified earlier (median age; 8 months) than 11 patients without laterality defects (median age; 12 years). Twelve (85.7%) patients were born to consanguineous parents. Neonatal respiratory distress was present in 10 (71.4%) PCD patients. While all patients had a wet cough, nasal and

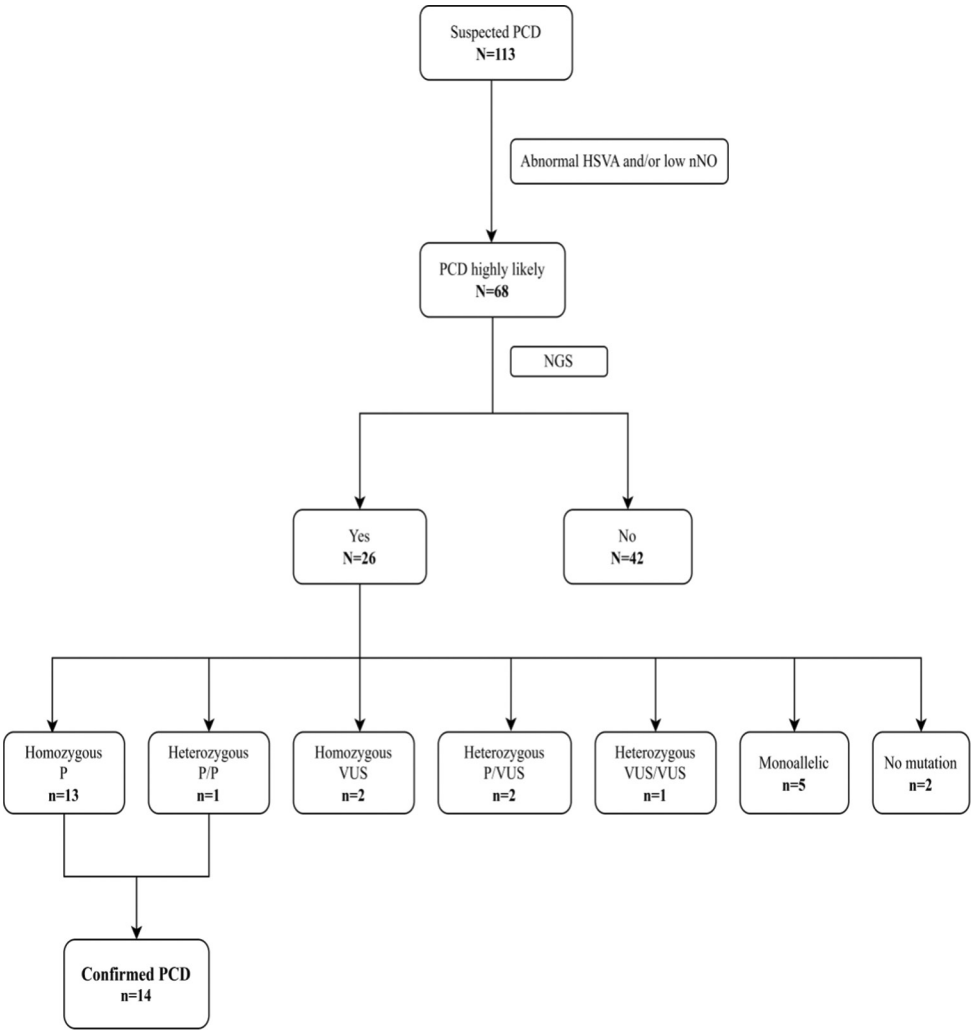

Figure 1: Flow chart diagram outlining the study patients. PCD = primary ciliary dyskinesia; HSVA = high-speed video microscopic analysis; nNO = nasal nitric oxide; NGS = next-generation sequencing; VUS = variants of uncertain significance; *P* = pathogenic

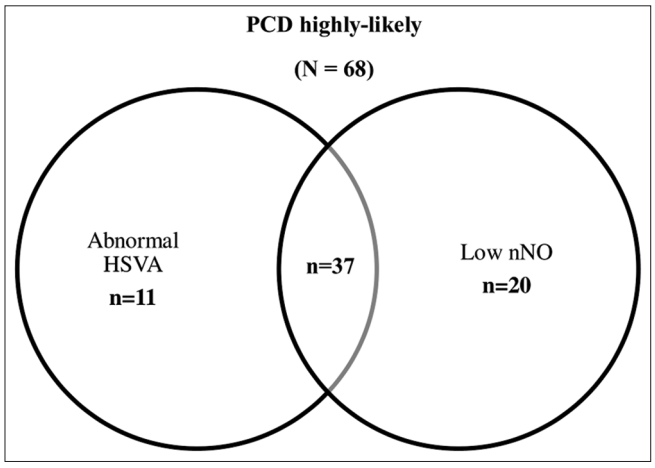

Figure 2: Venn diagram showing patients identified with PCD screening tests. HSVA = high-speed video microscopic analysis. nNO = Nasal Nitric oxide

ear symptoms were present in 11 (78.6%) and 4 (28.6%), respectively. At diagnosis, 8 (57.1%) patients had bronchiectasis.

DISCUSSION

In a resource-limited setting, cross-centre collaboration and targeted screening of patients with a clinical phenotype of PCD enabled the successful identification of individuals highly likely to have the disease. Screening with high-speed video microscopy analysis (HSVA) and nasal nitric oxide (nNO) allowed us to initiate timely clinical management.

HSVA is a sensitive, specific, and reliable test requiring technical expertise.[32] Before setting up our diagnostic service, we prioritised formal training with an experienced international team, which was key to successful implementation. Video conferencing platforms facilitated periodic interaction with the established PCD centre in Southampton for ongoing training, quality control, and multidisciplinary team discussions. We performed nasal brushings in 113 patients, and a conclusive HSVA outcome (normal or abnormal ciliary beat pattern) was obtained in 87 (76.9%) patients. Amirav *et al*.[15] reported a similar interpretability rate of 73% for HSVA in their

Table 1: PCD cohort genetics, high-speed video microscopy, and nasal nitric oxide

Patient	Gene	Variant	Type of mutation	Mutation state	ACMG	HSVM	nNo (ppb)
P1	CCDC39	c.1934C>T	Base pair deletion	Homozygous	P	Stiff beating	5
P2-1	CCNO	c.248_252dup	Duplication	Homozygous	P	Absent cilia	30
P2-2	CCNO	c.248_252dup	Duplication	Homozygous	P	Absent cilia	48
P3	DNAH11	c.9880C>T	Stop codon	Homozygous	P	Stiff beating	89
P4	DNAH1	c.5624_5625del	Frameshift	Homozygous	LP	Focal stiff beating	41
P5	DNAH11	c.9483+2T>A	Splicing	Heterozygous	LP	Stiff beating	18
	DNA H11	c.12748A>T	Stop codon	Heterozygous	LP		
P6	DNAH5	c.10872+1G>T	Splicing	Homozygous	LP	Immotile cilia	12
P7	DRC1	c.1205G>A	Nonsense	Homozygous	P	Stiff beating	12
P8-1	HYDIN	c.2851C>T	Nonsense	Homozygous	P	Stiff and rotatory motility	71
P8-2	HYDIN	c.2851C>T	Nonsense	Homozygous	P	Stiff and rotatory motility	44
P9	CCNO	c.258_262 dup	Duplication	Homozygous	P	Absent cilia	ND
P10	DNAAF2	c.932C>A	Stop codon	Homozygous	P	Immotile cilia	ND
P11	DNAH5	c.2109	Deletion	Homozygous	P	Immotile cilia	ND
P12	TTC25	c.1273C>T	Nonsense	Homozygous	P	Immotile cilia	ND

PCD, primary ciliary dyskinesia; HSVM, high-speed video microscopy; nNO, nasal nitric oxide; ACMG, American College of Medical Genetics; ND, not done

Table 2: Clinical features of confirmed PCD patients

Characteristics (*n*=14)	
Median age at diagnosis	8.5 years (3 months, 30 years)
Median age at diagnosis in patients without laterality defects (*n*=11)	12 years (3.4 years, 30 years)
Median age at diagnosis in patients with laterality defects (*n*=3)	8 months (3months, 4 years)
Gender (Male)	9 (52.9%)
Consanguinity	12 (85.7%)
Neonatal respiratory distress	10 (71.4%)
Nasal symptoms	11 (78.6%)
Ear symptoms	4 (28.6%)
Bronchiectasis	8 (57.1%)
Laterality defects	3 (21.4%)

PCD: primary ciliary dyskinesia

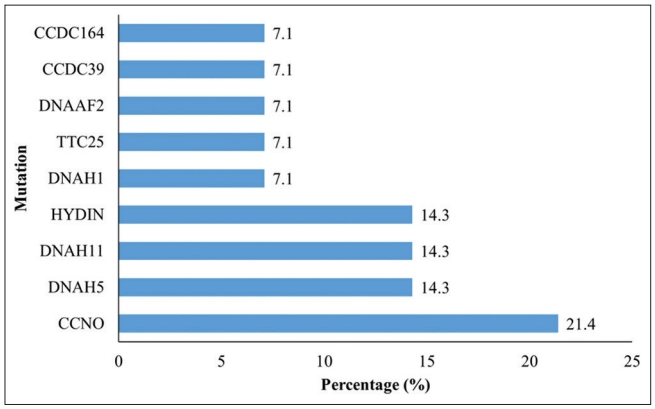

Figure 3: Percentage distribution of affected genes in confirmed PCD patients

cohort. Notably, HSVA could be performed across all age groups. Once the initial equipment was acquired, the test proved cost-effective and allowed for same-day results, enabling clinical decisions and justifying the cost of subsequent genetic testing.

The European Respiratory Society (ERS) PCD guidelines recommend nNO measurement as a screening tool for PCD≥≥. Although stationary chemiluminescence analysers are the gold standard, electrochemical analysers offer a practical alternative with lower cost and greater portability.[18] Their limitation lies in lower sensitivity and precision at very low NO levels. In our cohort, the use of both HSVA and nNO proved complementary—HSVA was useful in young children unable to perform nNO manoeuvres, and nNO provided diagnostic support in cases where HSVA was inconclusive.

PCD is genetically heterogeneous, with over 50 causative genes identified. *DNAH5* and *DNAH11* mutations are commonly reported worldwide.[33] In a study from Delhi, Jat *et al*.[34] identified mutations in *DNAAF11*, *DNAH5*, *CCDC39*, and *HYDIN* among Indian patients. In our cohort, *DNAH5*, *DNAH11*, and *CCNO* were the most common genes affected, with biallelic pathogenic or likely pathogenic variants in 7 (50%) of our genetically confirmed cases. Larger multicentre studies are necessary to better understand the genetic landscape of PCD in India's diverse population.

Consanguinity is a known risk factor for autosomal recessive diseases like PCD. In our study, 12 of the 14 genetically confirmed patients (85.7%) were born to consanguineous parents. O'Callaghan *et al*.[35] reported that 10 out of 19 (52.6%) British Asian patients with PCD had consanguineous parentage, highlighting the influence of familial structure on PCD prevalence.

The median age of diagnosis in our cohort was 8.5 years, reflecting a substantial delay. Importantly, two-thirds of these patients had already developed bronchiectasis by the time of diagnosis. Delayed recognition is likely due to limited awareness among clinicians and the lack of access to specialised diagnostic services.

Only 3 of our 14 PCD patients (21.4%) had laterality defects. This lower proportion may reflect regional genetic variation or missed referrals for patients with situs anomalies. Unexplained neonatal respiratory distress in term neonates—with or without situs inversus—is an important early marker of PCD.[36,37] In our study,

10 (71.4%) of the PCD patients had a history of neonatal respiratory distress. Chronic wet cough was universal, persistent nasal discharge was reported in 11 (78.6%) patients, and ear symptoms were observed in 4 (28.6%). These findings align with previously reported symptom frequencies in PCD cohorts.[38] The lower reported prevalence of ear symptoms in our PCD cohort may be due to under-reporting. Therefore, all patients should undergo objective evaluation for hearing loss and receive treatment if necessary.

Given the diagnostic delay and frequent presence of bronchiectasis at diagnosis, raising awareness is critical. Situs anomalies, neonatal respiratory distress, and persistent wet cough are key early warning signs. Greater awareness among neonatologists, cardiologists, and pediatricians is essential to facilitate early referral. Expanding the number of diagnostic centres and leveraging international networks will improve early diagnosis in resource-constrained settings.[9]

This study has several limitations. HSVA and nNO testing were performed only once in most patients due to cost and the difficulty of repeated travel for families from remote areas. While chemiluminescence remains the gold standard for nNO measurement, we used the electrochemical NIOX VERO® analyser for its affordability. Genetic testing could be performed only in a limited number of patients due to high cost. Additionally, we did not have access to transmission electron microscopy (TEM) or immunofluorescence (IF). IF, which detects the presence, absence, or mislocalisation of ciliary proteins, is a valuable diagnostic adjunct when TEM is unavailable and warrants further evaluation for use in our setting.[39]

CONCLUSION

In this resource-limited setting, cross-centre collaboration enabled the successful screening of patients with suspected PCD using HSVA and nasal nitric oxide (nNO) testing. Both modalities were found to be complementary in identifying screen-positive cases. Genetic testing, performed in a subset of these patients, confirmed the diagnosis in only 54%, reflecting the current limitations of genetic panels and incomplete knowledge of PCD-associated mutations. The median age at diagnosis was 8.5 years, indicating a significant delay. Alarmingly, over half of the confirmed cases had already developed bronchiectasis by the time of diagnosis, likely due to under-recognition of the disease and limited access to specialised diagnostic facilities.

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Contributions

Antony Terance Benjamin: conceptualization (lead); methodology (lead); data curation (equal); formal analysis (equal): writing – original draft (equal); writing – review and editing (equal). **Ramganesh**: Methodology (supporting); writing – review and editing (equal). **Madhumitha K**: conceptualization (supporting); writing – original draft (equal); formal analysis (equal); writing – review and editing (supporting). **Madhumitha Subramanium:** writing – original draft (equal); formal analysis (equal); writing – review and editing (supporting). **Kalyanasundaram Muthuswamy**: writing – review and editing (equal). **Vijayakumar Raju**: writing – review and editing (equal). **Claire L Jackson**: validation (equal); writing – review and editing (equal). **Janice Coles**: validation (equal); writing – review and editing (equal). **Sharada Iyer**: writing – review and editing (equal). **Jacinta D'souza**: writing – review and editing (equal). **Jane S Lucas**: conceptualization (supporting); supervision; validation (equal); writing – review and editing (equal).

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Conflicts of interest

NIOX VERO® equipment was received for the study as a research grant from Circassia, Sweden.

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