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## Clinical utility of NGS diagnosis and disease stratification in a multi-ethnic primary ciliary dyskinesia cohort

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## ABSTRACT

**Background:** Primary ciliary dyskinesia (PCD), a genetically heterogeneous condition enriched in some consanguineous populations, results from recessive mutations affecting cilia biogenesis and motility. Currently, diagnosis requires multiple expert tests.

**Methods:** The diagnostic utility of multi-gene panel next-generation sequencing (NGS) was evaluated in 161 unrelated families from multiple population ancestries.

**Results:** Most (82%) families had affected individuals with biallelic or hemizygous (75%) or single (7%) pathogenic causal alleles in known PCD genes. Loss-of-function alleles dominate (73% frameshift, stop-gain, splice site), most (58%) being homozygous, even in non-consanguineous families. Although 57% (88) of the total 155 diagnostic disease variants were novel, recurrent mutations and mutated genes were detected. These differed markedly between White European (52% of families carry *DNAH5* or *DNAH11* mutations), Arab (42% of families carry *CCDC39* or *CCDC40* mutations) and South Asian (single *LRR6* or *CCDC103* mutations carried in 36% of families) patients, revealing a striking genetic stratification according to population of origin in PCD. Genetics facilitated successful diagnosis of 81% of families with normal or inconclusive ultrastructure and 67% missing prior ultrastructure results.

**Conclusions:** This study shows the added value of high-throughput targeted NGS in expediting PCD diagnosis. Therefore, there is potential significant patient benefit in wider and/or earlier implementation of genetic screening.

## INTRODUCTION

Primary ciliary dyskinesia (PCD) is a rare genetic disease caused by cilia dysmotility that is associated with a range of defects of motile cilia structure and biogenesis. PCD is typically an autosomal or X-linked recessive disorder, caused by mutations in >40 different genes encoding structural ciliary proteins, cilia assembly and transport factors and proteins implicated in multi-ciliogenesis[1, 2]. Children and adults affected by PCD consequently manifest with progressive respiratory disease characterized by bronchiectasis and impaired lung function. Symptoms often present in early life with neonatal respiratory distress syndrome and persist with chronic wet cough, rhinitis, sinusitis, otitis media and hearing defects[3]. Defective cilia of the brain ependyma, fallopian tubes, and developing embryo can explain other disease features. Half of patients have laterality defects arising from embryonic nodal cilia dysfunction and a significant proportion of males are subfertile with defective sperm flagella. Affected individuals, in particular those with reduced cilia numbers, can also manifest with hydrocephalus, while *RPGR* and *OFDI* mutations can respectively cause rare retinal dystrophy and oral-facial-digital syndrome PCD subtypes[1, 2, 4, 5].

The prevalence of PCD is around 1:15,000 worldwide. PCD occurs much more frequently in highly consanguineous communities such as the UK South Asian population, in whom disease prevalence is as high as 1:2,265[3]. Generally, PCD symptoms are variable and diagnosis is frequently delayed or missed[6]. Early diagnosis has potential to improve morbidity since lung damage can be delayed by specialist care[3, 7]. PCD diagnostic testing requires access to a combination of investigations including measurement for low nasal nitric oxide levels, high

speed video microscopy for ciliary beating defects, ciliary ultrastructure defects analysed by transmission electron microscopy (TEM), immunofluorescence staining for abnormal motile cilia proteins and, increasingly, genetic analysis[7, 8].

PCD genetic diagnosis requires the identification of biallelic autosomal or hemizygous X-linked mutations[7, 8]. Mutations in known PCD genes are found in 60-70% of tested PCD patients[1, 3]. With additional genes still to be identified, the sensitivity of genetic testing as a ‘gold standard’ diagnostic test is reduced. However, with progressive identification of the whole ‘morbid genome’ causing PCD and ongoing reductions in DNA sequencing costs, genetics can increasingly be considered as a first line test in the diagnostic pathway. Gene panels can currently be more effective for target sequence coverage and reduced time and costs, than whole exome or genome sequencing[9].

Here, we present a targeted next generation sequencing (NGS) gene panel approach for characterization of a multi-ancestry cohort of PCD patients. Our aim was to investigate the utility of this approach for PCD, a clinically and genetically heterogeneous condition, where current diagnosis requires multiple expert tests[8].

## **MATERIALS AND METHODS**

### **Subjects**

161 unrelated families confirming self-reported ancestry and consanguinity at time of recruitment were ascertained from UK national PCD diagnostic and management services (London Royal Brompton Hospital, University Hospital Southampton, Leeds General Infirmary, Bradford Royal Infirmary, Birmingham Children's Hospital and Leicester Royal Infirmary) and collaborating clinical centres in Portugal (Hospital de Santa Maria, Centro Hospitalar Lisboa Norte, Lisbon), Palestine (Makassed Hospital, East Jerusalem) and Egypt (Alexandria University Children's Hospital, Alexandria). Recruitment took place between 01/2015-02/2017 with informed, age-appropriate consent as approved by the London-Bloomsbury Research Ethics Committee (08/H0713/82) and committees of collaborating institutions. The diagnosis of PCD followed the European Respiratory Society (ERS) guidelines[8], using various methods according to the clinical centre, including clinical presentation and the results of formal PCD diagnostic tests (nasal NO level, cilia ultrastructure analysis by transmission electron microscopy, cilia beat pattern and frequency by high speed video microscopy and immunostaining against specific ciliary proteins). Study inclusion criteria was based upon a clinical suspicion of PCD and/or available cilia ultrastructural TEM analysis. TEM data was not available in a total of 27 families, who were included to the study based upon other clinical criteria suggesting PCD.

## **Targeted next generation sequencing (NGS)**

Genomic DNA extracted from whole blood samples or saliva was screened for mutations using targeted NGS gene panels containing all the known PCD (**online supplementary table S1**) and isolated heterotaxy genes, plus one of two iterations of a larger set of cilia motility associated candidate genes. These were collated after extensive literature searches for candidates with cilia involvement confirmed or likely and from data from previous human genetics and PCD model organisms studies. Panel probe design used the Agilent SureDesign tool (Agilent Technologies, Santa Clara, CA, USA) to capture all coding regions and 25 bp at the exon-intron boundaries (**online supplementary tables S1 and S2**). Capture probes were enriched in regions with potential low coverage. Library preparation used the SureSelectQXT NGS target enrichment kit (Agilent Technologies, Santa Clara, CA, USA) was used for library preparation following the manufacturer's protocol. Paired end sequencing (2 x 150bp) was performed using the NextSeq 500/550 High Output v2 kit and NextSeq sequencing platform (Illumina, Inc., CA, USA). Multiplexing of 48 samples was done on the same flow cell per sequencing run. Sequencing data were processed using an in-house bioinformatics pipeline at North East Thames Regional Genetics Service[10]. Variants were filtered for significance to produce variant lists of interest in each patient that conform to the expected minor allele frequency for PCD (<1%) and an autosomal or X-linked recessive inheritance pattern. Variants were prioritized based on their minor allele frequency in the ExAc database[11], 1,542 individuals in the Born-in-Bradford (BinB) cohort of UK South Asians[12] and the al mena database of genetic variants in Middle East and North African individuals[13]. Potential pathogenicity was assessed using several softwares including Human Splicing Finder, SIFT, Polyphen-2, Mutation Taster and Combined Annotation Dependent Depletion (CADD) score. Variant



pathogenicity scoring was done according to the guidelines of the American College of Medical Genetics and Genomics (**online supplementary figure S1**)[14], using a well-established classification (or tiering) system of predicted (i) pathogenic, (ii) likely pathogenic, (iii) uncertain significance, (iv) likely benign, or (v) benign variants. Details of all variants and gene transcript numbers are contained in **online supplementary table S3**. For all affected individuals, a search for large insertion/deletion mutations and copy number variants (using ExomeDepth software) was separately performed[15].

### **Sanger Sequencing**

All prioritized variants were confirmed in the proband and segregated within the available family members using Sanger sequencing. Sequencing data was viewed using SnapGene (GSL Biotech LLC, Chicago, USA) or Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA).

## **RESULTS**

### **Targeted NGS yields high diagnostic output in a multi-ancestry cohort of PCD patients**

The probands from 161 unrelated families were screened using targeted NGS multi-gene panel analysis, followed by Sanger sequencing-based segregation analysis to confirm all identified genetic variants of interest and determine their familial inheritance pattern. All families had affected individuals with a suggestive clinical phenotype, in addition to either (1) a ciliary

ultrastructural defect confirmed (97 families); or (2) inconclusive TEM results where PCD was still highly suggestive (37 families); or (3) no diagnostic TEM analysis yet performed but PCD still clinically highly suspected (27 families). Their details are summarized in **table 1**. The PCD-consistent features of the total 27 families with probands lacking TEM data are summarized in **online supplementary table S4**. The ancestry and consanguinity of all the families are summarized in **online supplementary table S5**, showing that 46% (74) were European, 22% (35) South-Asian, 18% (29) Arab and the rest had other ancestries. Consanguinity was reported in 29% of 161, with highest levels in the Arab (25; 86%) and South-Asian (12; 34%) families.

TEM ultrastructural phenotype Total no. families (% of 134 with TEM defects defined)	Mutant known PCD gene or novel candidate	No. families
Normal TEM and inconclusive TEM analysis (No apparent defect, or few observed defects insufficient to make a diagnosis) 37 families (28%)	<i>DNAH11</i> †	15
	<i>HYDIN</i> †	6
	<i>DNAH5</i>	3
	<i>CCDC103</i> †	2
	<i>RSPH1</i>	1
	<i>OFD1</i>	1
	<i>DNAI2</i>	1
	<i>ZMYND10</i>	1
	Novel candidate gene	4
	Unsolved	3
Outer dynein arm loss 31 families (23%)	<i>DNAH5</i>	17
	<i>DNAI1</i>	3
	<i>ARMC4</i>	2
	<i>DNAI2</i>	1
	<i>CCDC151</i>	1
	<i>SPAG1</i>	1
	<i>PIH1D3</i>	1
	Unsolved	5
Combined inner and outer dynein arm loss 30 families (22%)	<i>LRRC6</i>	5
	<i>DNAAF3</i>	5
	<i>CCDC103</i>	3
	<i>DNAH5</i>	3
	<i>ZMYND10</i>	2
	<i>DYX1C1</i>	1
	<i>DNAAF1</i>	1
	<i>HEATR2</i>	1
	<i>DNAI1</i>	1
	Novel candidate gene	4*
Unsolved	4	
Microtubular disorganization ± inner dynein arm loss 16 families (12%)	<i>CCDC40</i>	7
	<i>CCDC39</i>	5
	<i>CCDC65</i>	1
	<i>RSPH9</i>	1
	<i>RSPH1</i>	1
	Unsolved	1
Central microtubular pair defect 8 families (6%)	<i>RSPH4A</i>	4
	<i>RSPH1</i>	2
	<i>RSPH9</i>	1
	Unsolved	1
Inner dynein arm loss 5 families (4%)	<i>CCDC103</i>	2
	<i>CCDC164</i>	1
	<i>CCDC40</i>	1
	Novel candidate gene	1**
Lack of cilia cross sections 7 families (5%)	<i>CCNO</i>	2
	<i>MCIDAS</i>	2
	<i>DYX1C1</i>	1
	<i>RPGR</i>	1
	Novel candidate gene	1
No TEM analysis 27 families	<i>CCDC40</i>	5
	<i>DNAH5</i>	4
	<i>CCDC39</i>	3
	<i>LRRC6</i>	2
	<i>CCDC114</i>	1
	<i>RSPH9</i>	1
	<i>DNAH11</i>	1
	<i>ZMYND10</i>	1
	Novel candidate gene	2
	Unsolved	7

**Table 1. Genetic stratification of 161 unrelated PCD families, according to transmission electron microscopy findings.** Mutations in genes regarded during the study as candidates but since published as PCD genes were found in \*2 families with *CFAP300* and 1 family with *DNAH9* variants; \*\*1 family with *DNAH9* variants [16, 17]. †*DNAH11*, *HYDIN* and often *CCDC103* mutations are associated with normal TEM, whilst the other genes in this group are associated with visible TEM defects [18-20].

We identified causal variants in known PCD genes in 128 of the 161 PCD families, comprising 82% of the cohort (**figure 1A and online supplementary table S3**). Biallelic autosomal or hemizygous X-linked variants were identified in 116 families in known PCD genes and in a further 4 families in genes considered during the study to be candidate genes, comprising two families with *CFAP300* variants[16] and two with *DNAH9* variants[17]. Since these genes have been recently verified as PCD-causing, this means that a total of 120 out of 161 families (75%) were diagnosed. In 12 families (7%), only one mutant allele (single heterozygous) was found in a known autosomal PCD gene, which is considered an incomplete genetic diagnosis, however we include the variant data for all 12 families, since seven are protein truncation variants and 5 are already reported in previous studies on PCD patients; also, all these ‘single hit’ variant carrying patients had cilia ultrastructural defects consistent with the implicated mutant gene (**figure 1A and online supplementary table S3**).

For 13 families (8%), biallelic variants in candidate genes for PCD (*CFAP300*, *DNAH9* included at the time) were identified and further functional characterization of these genes and their roles in causing PCD are ongoing. Finally, 20 families (12%), had no putative significant sequence variants, 11 of these having cilia ultrastructural defects identified by TEM and 3 with low nasal nitric oxide and abnormal cilia beat frequency but inconclusive TEM (**online supplementary figure S2**), the other 6 having a strong clinical suspicion of PCD (situs inversus and recurrent respiratory problems) without prior investigations.

## Significant, population-based genetic stratification underlies PCD

Prioritized variants for the 128 diagnosed families were identified within different functional categories of known PCD genes (**figure 1B**). The most prevalent, identified in 38% of families, affected genes encoding outer dynein arm (ODA) components (*DNAH5*, *DNAH11*, *DNAI1*, *DNAI2*). The second collectively most common affected genes encoding dynein assembly factors (*LRRC6*, *DNAAF3*, *ZMYND10*, *DYX1C1*, *DNAAF1*, *PIH1D3*, *SPAG1*, *HEATR2*) in  $\approx 17\%$  of families, followed by mutations in ‘ruler protein’ genes (*CCDC39*, *CCDC40*) in 16% and radial spokes (*RSPH1*, *RSPH3*, *RSPH4A*, *RSPH9*) in 8%. *CCDC103* mutations affected 5% families but otherwise, mutations in gene involved in ODA docking (*ARMC4*, *CCDC114*, *CCDC151*), central pair (*HYDIN*) and nexin-dynein regulatory complex structures (*CCDC65*, *DRC1*), multi-ciliogenesis (*CCNO*, *MCIDAS*) or causing ‘syndromic’ forms (*OFD1*, *RPGR*) were more rare, affecting collectively  $\approx 9\%$  of the families.

Overall, *DNAH5* was the most prevalent mutant gene, mutations identified in affected individuals from 27 families (21%) (**figure 1B and detailed in figure 2A**). However, populations of different ancestry (ethnicity) had considerably different genetic profiles. *DNAH5* and *DNAH11* mutations were found in 37% and 15% of European families respectively, but in only a minority of patients from other ancestries. *LRRC6* and *CCDC103* were the most frequently mutated genes in South-Asian families, affecting overall more than a third (20% and 16% respectively) of families. *CCDC39* and *CCDC40* were the major two mutant genes affecting the Arab population, identified in 42% of Arab families (**figure 1C**). As detailed further below, these frequencies were due to a mixture of recurrent, presumed founder effect mutations, as well as mutations often unique to individual families.

### Expanded mutation spectrum in known PCD genes

A high proportion of families (74/128, 58%), from all ancestry groups, were found to carry homozygous variants (**figure 2B**). Surprisingly, one third of European families (20/60) considered largely non-consanguineous, carry homozygous variants in known PCD genes (**online supplementary figure S3**), highlighting possible unrecognized endogamy and relatedness. Biallelic heterozygous variants in autosomal genes caused disease in 31% of patients (39 families) and in 3 families, hemizygous variants were identified in known X linked genes (*PIH1D3*, *RPGR*, *OFD1*).

Of the total of 167 variants in known PCD genes detected in the 128 families, the predominant variant types were predicted protein truncating mutations (73%) classified as frameshift 32%, nonsense 26%, and mutations affecting splicing 15%. Missense variants accounted for 21% of all variants. Copy number variations (CNV) and in-frame deletions or deletion/insertion mutations accounted for 6% of variants overall (**figure 2C**). Eleven single variants identified without a second mutation ('one hit' patients in **table S4**) were not regarded as diagnostic, but amongst the 155 variants that diagnosed 116 families (excludes *DNAH9* and *CFAP300* alleles), 82% were pathogenic (class 5) and 8% were likely pathogenic (class 4). Class 3 variants of unknown clinical significance (VUS) represented only 10% of variants; these remain under some caution for providing a definitive diagnosis (**figure 2D**).

## Marked differences in the frequency and spectrum of mutations in different ancestries

Across the cohort, in addition to the presence of many family-unique mutations, the prevalence of a number of recurrent mutations presumed to reflect population bottleneck/founder effects, play a major contribution to the different genes affecting different PCD populations. We defined 14 recurrent mutations that collectively accounted for a large number of the PCD-causing variants. Some were ancestry-specific and others were present in multiple populations (**table 2**). In the 60 European families where causal alleles were defined, three recurrent *DNAH5* mutations were identified to account for 13% (16/120) of European disease alleles, two previously reported as possible founder effects (c.10815delT; p.Pro3606Hisfs\*22 and c.13458\_13459insT; p.Asn4487fs\*1)[20-22] and a nonsense mutation (c.6261T>G; p.Tyr2087\*) not previously reported (table 2, online supplementary table S3 and figure 2A). Three other previously reported mutations together accounted for another 13% (16/120) of European disease alleles: *DNAH11* c.48+2dupT; p.Ser17Valfs\*12, *RSPH1* c.275-2A>C; p.Gly92Alafs\*10 and a recurrent homozygous *DYX1C1* 3.5 kb genomic deletion[23-26]. *DNAH11* mutations are also a major contributor to European PCD disease, but mostly as family-unique rather than recurrent alleles.

Mutation	No. of alleles/ alleles per ancestry	No. of alleles/total alleles	Ancestry (ethnicity)	ExAc_M AF	al mena_MAF	BinB_MAF	Reported before
<i>CCDC39</i> (c.1871_1872del, p.Ile624Lysfs*3)	8/48 17%	8/218 4%	Arab	Not in ExAc	Not in al mena	Not in BinB	No
<i>RSPH9</i> (c.801_803delGAA, p.Lys268del)	4/48 8%	4/218 2%	Arab	0.0000576 5	3 / 0.00151	Not in BinB	Yes
<i>DNAH5</i> (c.10815delT, p.Pro3606Hisfs*22)	8/120 7%	8/218 4%	European	0.0001483	Not in al mena	Not in BinB	Yes
<i>DNAH5</i> (c.13458_13459insT, p.Asn4487fs*1)	5/120 4%	5/218 2%	European	0.0000578 3	Not in al mena	Not in BinB	Yes
<i>DNAH5</i> (c.6261T>G, p.Tyr2087*)	3/120 2%	3/218 1%	European	Not in ExAc	Not in al mena	Not in BinB	No
<i>DNAI1</i> (c.48+2dupT, p.Ser17Valfs*12)	5/120 4%	5/218 2%	European	0.0004624	Not in al mena	Not in BinB	Yes
<i>RSPH1</i> (c.275-2A>C, p.Gly92Alafs*10)	7/120 6%	7/218 3%	European	0.0003625	Not in al mena	Not in BinB	Yes
<i>DYX1C1</i> (3.5kb del involving exon 7)	4/120 3%	4/218 2%	European	NA	NA	NA	Yes
<i>LRRC6</i> (c.630delG, p.Trp210Cysfs*12)	10/50 20%	10/218 %	South- Asian	0.000206	Not in al mena	10/0.0065	Yes
<i>CCDC103</i> (c.383dup, p.Pro129Serfs*25)	4/50 8%	4/218 5%	South- Asian	Not in ExAc	Not in al mena	1/0.0006	Yes
<i>CCDC103</i> (c.461A>C, p.His154Pro)	8/218 4%	8/218 4%	Multiple ancestries	0.001261	2 / 0.00101	6/0.0039	Yes
<i>CCDC40</i> (c.248delC, p.Ala83Valfs*84)	4/218 2%	4/218 2%	Multiple ancestries	0.0004794	Not in al mena	Not in BinB	Yes
<i>CCDC40</i> (c.2824_2825insCTGT, p.Arg942Thrfs*57)	3/218 1%	3/218 1%	Multiple ancestries	Not in ExAc	Not in al mena	Not in BinB	No
<i>DNAH11</i> (c.13494_13500del, .Ser4498Argfs*15)	3/218 %	3/218 %	Multiple ancestries	Not in ExAc	Not in al mena	Not in BinB	No

**Table 2. Ancestry-specific frequent mutations.** No. of alleles is calculated for the 109 families in which causal alleles were identified (60 European, 25 South-Asian and 24 Arabic ancestry families). MAF, minor allele frequency; ExAc[11], Born-in-Bradford (BinB)[12] and al mena[13] databases.



In South-Asian families, a previously described *LRRC6* mutation (c.630delG; p.Trp210Cysfs\*12) was the most frequent mutant allele, found in homozygous status in five South-Asian families[21]. A previously reported *CCDC103* mutation was detected in homozygous state (c.383dupG; p.Pro129Serfs\*25) in two unrelated South-Asian families[22]. Together, these two variants alone accounted for 28% (14/50) of all disease alleles in the 25 South-Asian families where causal alleles were defined (**table 2 and online supplementary table S3**). A known recurrent Arabic Bedouin *RSPH9* mutation (c.801\_803delGAA; p.Lys268del) was detected in homozygous state in two Arab families[23]. Another possible Arabic homozygous founder mutation (c.1871\_1872delTA; p.Ile624Lysfs\*3) in *CCDC39* was found in 4 Palestinian families. Together, these two variants accounted for 29% (12/48) of all disease alleles in the 24 Arabic families where causal alleles were defined (**table 2 and online supplementary table S3**). *CCDC40* mutations also contribute to Arabic PCD disease, but in the form of family-unique rather than recurrent alleles.

Of other recurrent variants, the previously reported common South-Asian *CCDC103* missense mutation (c.461A>C; p.His154Pro) was detected mostly in South-Asians, but also in European and other ancestries[18]. We also identified in different ancestries two recurrent *CCDC40* mutations, one previously reported (c.248delC; p.Ala83Valfs\*84)[24, 25] and one novel (c.2824\_2825insCTGT; p.Arg942Thrfs\*57), in addition to one novel *DNAH11* mutation (c.13494\_13500del, p.Ser4498Argfs\*15).

## **Targeted NGS reveals synonymous variants predicted to affect splicing as a cause of PCD**

We identified two synonymous coding region variants not predicted to change the encoded protein's amino acid sequence, but predicted instead to affect splicing. One in an Arab family (PCD-G086) with cilia microtubular disorganization and IDA loss, was a *CCDC40* homozygous variant (c.48A>G; p.Gly16Gly) that correctly segregated within the extended family (**online supplementary figures S4, S5**). The other in a European family (PCD-G093) with cilia ODA loss, was a *DNAH5* synonymous mutation (c.5157C>T; p.Phe1719Phe) that was combined with a missense variant (c.10815T>G; p.Asp3605Glu) (**online supplementary figures S6, S7**). Whilst it is possible these synonymous variants may affect splicing, they are currently class 3 VUS (**online supplementary table S3**) and cannot be reclassified to pathogenic or likely pathogenic without further work that provides direct observation of their presumed splicing effects.

## **Targeted NGS is a powerful tool for diagnosis and characterization of PCD patients**

TEM analysis detected ultrastructural defects in 97/134 (72%) families. The other 37 had either normal TEM (e.g. associated with *DNAH11* and *HYDIN* defects[19, 20] or a minority of inconclusive TEM results (**table 1**). The most common ultrastructural defect was ODA loss (in 45%, 61 families), either alone (in 23%, 31 families) or combined with IDA loss (in 22%, 30 families). Other defects included microtubular disorganization with or without IDA loss (12%), central microtubular complex defects (6%), predominant isolated IDA loss (4%) or a lack of cilia (5%) (**table 1**).

We confirmed a strong correlation across the entire cohort between gene defect and expected ultrastructural defect, in agreement with the PCD literature (**tables 1 and online supplementary table S1**)[2]. Hence, TEM defects can be valuable for interpretation of genetics test results; however, the study also showed that they are not always required. For the 27/161 families (17%) without TEM data, still with strong clinical suspicion, 18 had biallelic variants in known PCD genes, hence a high proportion (67%) were confidently solved by genetics without TEM information (**online supplementary figure S8**). As a cautionary note, for a small number of patients (n=6, asterisked in **online supplementary table S3**) without a recorded TEM defect confirming of their PCD status, they also carry homozygous or biallelic heterozygous variants that are rare and in the known PCD genes, but are class 3 VUS of uncertain significance. For example PCD-G013 is biallelic heterozygous for two *DNAH5* missense changes both of unknown significance (not previously reported). In these cases, three have variants in the *HYDIN* and *DNAH11* genes associated with normal TEM (PCD-G104, -G017, -G021), but for the others the TEM could be reviewed and repeated.

To further test the power of genetic testing in the diagnostic workflow of PCD, we looked in detail at the correlation of specific PCD gene mutations with ciliary ultrastructural defects determined by TEM at a single diagnostic centre. We found that mutations in the ODA gene *DNAH5* were associated with (i) clear-cut ODA loss as expected, but also (ii) combined loss of IDA+ODA or (iii) inconclusive TEM analysis (**figure 3A; table 1**). A similar classification was possible in individuals with dynein assembly gene mutations (*LRRC6*, *HEATR2*, *DYX1C1*, *DNAAF3* or *DNAAF1*), where combined IDA+ODA loss is expected (**figure 3B; table 1**). By looking at the TEM data in the context of the genetic mutation, we could see a distinct pattern, since dynein assembly mutations led to combined IDA+ODA loss in most cilia cross sections,

contrasting with *DNAH5* mutations causing mainly ODA loss. Therefore, genetic data allows these two categories of genetically diagnosed patients to be distinguished (**figure 3**).

## **DISCUSSION**

There is high underlying disease heterogeneity and no gold standard test available yet to exclude PCD, so a combination of tests interpreted in the light of clinical symptoms tends to be used for diagnosis. This increasingly includes genetic analysis[8]. Here, the utility of genetic screening was evaluated in a large cohort of 161 unrelated PCD families from various ancestries including European, Arab and South-Asian, by NGS screening with additional CNV analysis of the known PCD genes and a panel of other candidate genes. This gave a high yield of a confirmed or highly suggested PCD diagnosis in 75% of families. A further 7% of families with single heterozygous variants in known PCD genes that looked likely causal likely may carry a second mutation that NGS was not able to detect, for example a deep intronic variant.

The identification of clear-cut ciliary ultrastructural defects by TEM analysis remains a confirmatory step in the PCD diagnostic workflow, although failure to identify TEM defects does not exclude PCD[8]. Here, we identified mutations in known PCD genes in 81% of patients with normal or inconclusive TEM findings, implying significant potential for incorporating genetics earlier within the diagnostic pipeline, as previously discussed[19]. We could also diagnose 67% of patients with a strong history where TEM was not available, as well as other difficult cases e.g. *CCDC103* p.His154Pro mutations, where other tests often give equivocal results[18].

Our diagnostic output is higher than most previous NGS targeted panel screens in PCD[26-29], similar to the 76% diagnostic success achieved from WES and targeted CNV analysis in 52 individuals[30, 31]. The limitations include the unknown genes that are absent from the panel, the incomplete genetic diagnosis when variants of uncertain significance or single heterozygous variants in PCD genes are detected, technical issues affecting sequence coverage depth, the known bioinformatics challenges to identify CNVs[32] and a well-known problem with identification of *HYDIN* mutations, due to the *HYDIN2* copy gene[20].

This study expands the genetic landscape and mutation spectrum of PCD by identifying 61 previously reported and 88 previously unrecognized variants, hence 57% of all the variants classified here as likely pathogenic are novel (**online supplementary table S3**). Most were protein truncating mutations, consistent with previous reports. Synonymous mutations are not commonly reported in PCD, but we identified two predicted to result in alteration of splicing, raising the importance of looking for potential synonymous variants in unsolved cases.

In agreement with previous studies, most of the identified PCD variants were private[1, 8], however several were detected in more than one unrelated family that tended to be more frequent in certain populations. Interestingly, one third of mutations in European families were homozygous despite the low recorded European consanguinity rate, with only one European family reporting consanguineous marriage. In 14 European families, their identified mutations were reported before in the literature, suggesting they may reflect European founder effects. Overall, we found that *DNAH5* is the most commonly mutated PCD gene in agreement with other studies[33], but this was not the case in all ancestries. We identified *DNAH5* mutations in 37% of European families, representing the most common mutant gene in Europeans. In

contrast, *LRR6* and *CCDC103* mutations were more prevalent in South-Asian families amongst whom we found only one family with *DNAH5* mutations. In Arab families, *DNAH5* mutations were identified in only two families, with *CCDC39*, *CCDC40* and *RSPH9* mutations much more prevalent.

The study has therefore uncovered a striking population-based genetic stratification underlying PCD. It highlights the impact of ancestry upon the genetics of PCD and the importance of including patients from various ancestries to elucidate the full genetic landscape of PCD. This information is diagnostically relevant, as it could be used for improved, smaller/cheaper carrier screening panels targeting certain populations and preliminary allelic-specific genetic diagnosis by Sanger sequencing, especially in countries where NGS facilities are not widely available. The clinical relevance of genetic disease stratification remains poorly understood, but more studies are emerging with PCD genotype-phenotype correlations which can increasingly impact upon disease management[4, 34-36].

Although we could confirm good correlation between genotype and cilia ultrastructural phenotype, some differences in the TEM analysis results were evident even with mutations in the same gene. *DNAH5* mutations were associated with ODA loss in the majority of cases but also with inconclusive TEM results, possibly due to difficulties in evaluating IDA by TEM[3, 8], as well as with combined IDA+ODA loss that is more often linked to dynein assembly gene mutations. Quantification of the percentage of arm loss arising from *DNAH5* mutations compared to mutations in dynein assembly genes showed that augmenting TEM data with genetics could clearly distinguish these two groups, *DNAH5* mutations being more highly

linked to ODA loss and dynein assembly mutations more highly to combined IDA and ODA loss.

In conclusion, targeted multi-gene panel sequencing is a cost-effective, time efficient single test which in this study diagnosed around 75% of PCD cases. It improves the diagnostic workflow outcome, confirming PCD in patients with inconclusive TEM results and helping in diagnosis of patients where TEM analysis is not available. The sensitivity (diagnostic yield) of gene tests for PCD will continue to increase with gene discovery progress. *CFAP300*, *DNAH9*, *GAS2L2*, *LRRC56*, *MNS1*, *DNAH1*, *DNAH6* are all genes that have become associated with PCD or confirmed as PCD genes, in the interim study period[16, 17, 37-41]. Despite the current incomplete PCD gene list, this strongly supports the importance of including genetics into the diagnostic pathway where it can play a key role, overcoming the pitfalls of other diagnostic measures. This may be particularly relevant in countries where access to other specialized PCD tests is not available. Major impact genes and recurrent mutations have emerged in this study, in addition to a notable impact of ancestry on the genetic variability of PCD, which has implications for the improved stratification of PCD patients to help facilitate better targeting of diagnostics and disease management.

## **CONTRIBUTORSHIP STATEMENT**

MRF, MPP, TC, JH, LJ, DM-R, CMW, HM and HMM performed genetic and bioinformatic analysis and analysed genetics data. AS, MD, AVR, SO, CJ, PG, RAH, AR, JT, AP and SL were involved in generating and analysing clinical functional tests. AS, MD, AVR, PG, AR and AP performed cilia ultrastructural analysis. MRF, PA, EM, PC, CO'C, RW, SC, WW, HM, WS, LP, CC, MRL, EMKC, PK, NR, NF, JSL, CH and HMM ascertained patients and acquired patient data and samples. HMM conceived and supervised the study and data interpretation. MRF interpreted the genetics data, developed the manuscript draft and generated the figures. MRF and HMM wrote the manuscript with critical review input from AS, TC, CMW, JSL and CH. All authors reviewed and approved the final manuscript.

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## **COMPETING INTERESTS**

The authors declare that they have no competing interests.

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## Figure legends

### **Figure 1. Targeted next generation sequencing yields a high (72%) diagnostic output in PCD patients and reveals a diverse mutation landscape stratified by ancestry**

A, Flow chart of genetic results found for families enrolled in the study describing the genetic diagnostic output. Probands from 161 unrelated families were subject to next generation sequencing using a multi-gene motile ciliome panel. Affected individuals in four of the 13 ‘candidate gene’ families carried biallelic variants in two genes now recognized as disease-causing, *CFAP300* and *DNAH9*, see main text for details. B, The genetic stratification of all 161 from the multi-ancestry cohort found to carry mutations in known PCD genes. C, Summary of mutated genes in three different populations illustrating that different ancestries have different genetics. Amongst 109 European, Arabic and South Asian families, the genes most commonly detected to carry mutations are *DNAH5* in European families (37%), *LRRC6* and *CCDC103* in South-Asian families (36%) and *CCDC39* and *CCDC40* in Arabic families (42%).

### **Figure 2. PCD-causing mutation distribution in the multi-ancestry cohort is dominated by protein truncating and homozygous mutations**

A, Schematic of the *DNAH5* mutations identified in this study, marked in yellow if previously reported. Recurrent mutations are boxed in bold. Conserved domains of the *DNAH5* protein are indicated on the genomic structure. Variants are numbered according to (NM\_001369.2) transcript. B, Families with mutations in known PCD genes grouped based on their zygosity status showed that about 58% of mutations identified in this study were present in patients in a homozygous state. C, Mutations classified according to their

impact on the respective proteins showed that frameshift and nonsense mutations were the most prevalent (58%) with 15% splicing defects and 21% missense changes. Collectively, mutations predicted to have a protein truncating effect represent about 77% (frameshift, nonsense, splicing defects and CNVs). D, Mutations identified in biallelic state in autosomal gene or hemizygous state in X-linked gene classified based on the guidelines from the American College of Medical Genetics showed that 82% of mutations were class 5 (clearly pathogenic), 8% class 4 (likely pathogenic) and 10% class 3 (uncertain significance).

**Figure 3. Genetics can better characterize PCD patients and overcome other diagnostic testing inconsistencies**

For a selected set of patients, the percentage of cilia cross sections showing a loss of either or both the inner and outer dynein arms was recorded in the routine TEM diagnostic setting at Royal Brompton Hospital with reference to the underlying genetic defects. a) Patients carrying biallelic mutations in outer dynein arm components showed mainly an isolated loss of outer dynein arms. Combined IDA+ODA loss was also noted. Interesting, quite variable numbers of cross sections showed a normal ultrastructure of the cilia. b) Patients carrying biallelic mutations in dynein assembly genes showed a combined loss of both arms in the majority of cross sections examined. Variable isolated arm loss was also reported.