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Unique among ciliopathies: primary ciliary dyskinesia, a motile cilia disorder

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Abstract

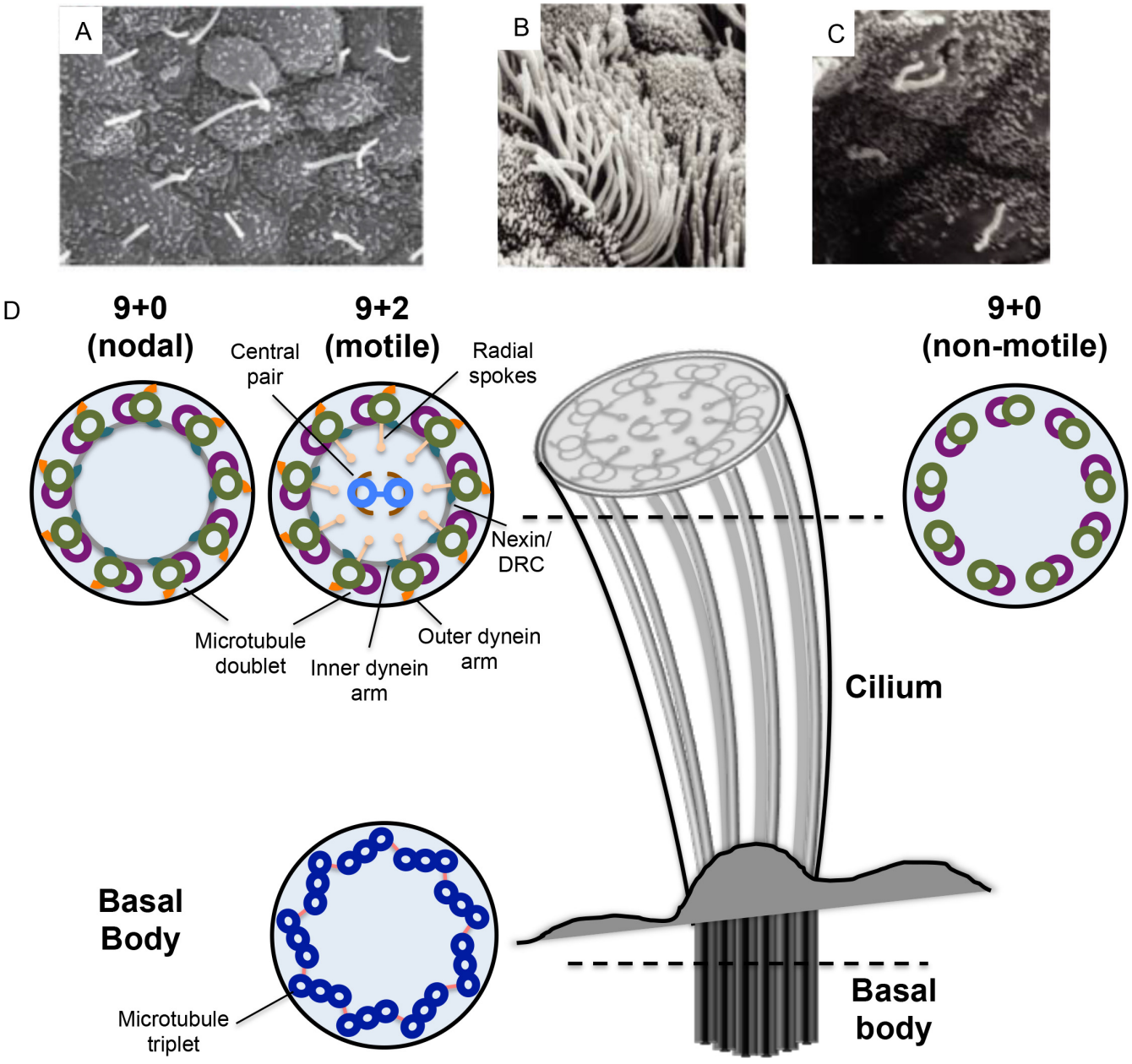
Primary ciliary dyskinesia (PCD) is a ciliopathy, but represents the sole entity from this class of disorders that results from the dysfunction of motile cilia. Characterized by respiratory problems appearing in childhood, infertility, and *situs* defects in ~50% of individuals, PCD has an estimated prevalence of approximately 1 in 10,000 live births. The diagnosis of PCD can be prolonged due to a lack of disease awareness, coupled with the fact that symptoms can be confused with other more common genetic disorders, such as cystic fibrosis, or environmental insults that result in frequent respiratory infections. A primarily autosomal recessive disorder, PCD is genetically heterogeneous with >30 causal genes identified, posing significant challenges to genetic diagnosis. Here, we provide an overview of PCD as a disorder underscored by impaired ciliary motility; we discuss the recent advances towards uncovering the genetic basis of PCD; we discuss the molecular knowledge gained from PCD gene discovery, which has improved our understanding of motile ciliary assembly; and we speculate on how accelerated diagnosis, together with detailed phenotypic data, will shape the genetic and functional architecture of this disorder.

Introduction

The cilium is a microtubule-rich organelle that protrudes from the apical membrane of most vertebrate cells (Figure 1). Observed initially via a light microscope over 300 years ago [1], and largely thought of as "vestigial" or "rudimentary" organelles, cilia garnered attention in 1976 because of their association with PCD (also known as Kartagener syndrome when associated with *situs inversus*) by Bjorn Afzelius [2]. He described four patients with chronic sinusitis, bronchitis, frequent ear infections and immotile sperm, three of whom displayed *situs inversus totalis* (reversal in placement of body organs). He observed a lack of ciliary motion in biopsy samples from bronchial mucosa of one patient, along with an absence of the normal numbers of dynein arms in ciliary electron micrographs [2]. The seemingly stationary cilia in these patients led to the initial naming of the disease as "immotile-cilia syndrome" [3]. Further investigation into the disorder, however, showed the cilia to have dysfunctional motility, rather than a lack of all movement; thus the clinical disorder was renamed primary ciliary dyskinesia [4].

Cilia can be found on cells within almost all organ systems in the body (Figure 1A, B, C). Historically, cilia have been divided into two types based on their ability to move: motile cilia and primary (non-motile) cilia (Figure 1D). While motile cilia achieved prominence through their connection to PCD, and their functions in cell movement (e.g. sperm) and flow generation (e.g. mucosa), the importance of primary cilia remained underappreciated for several years after their discovery. The first evidence of an important sensory function for primary cilia came from the discovery that the mutation underscoring the cystic renal phenotype of the *Tg737* mouse mutant was a hypomorphic allele in the gene encoding IFT88, a protein important for the assembly of flagella in *Chlamydomonas* [5]. Subsequently, it was

Figure 1. Overview of ciliary ultrastructure and comparison between motile and non-motile cilia



A. Scanning electron micrograph of mouse nodal cilia (image reproduced with permission from Shigenori Nonaka). B. Motile cilia are usually found at a density of several hundred per cell, as shown in this scanning electron micrograph of the mammalian trachea (image from [102]). C. Primary cilia are typically one per cell, as exemplified by primary cilia in renal tubule epithelia (image from [102]). D. Schematic representation of a cilium, with cross sections at the distal end showing the microtubule arrangements in the nodal, motile and non-motile (primary) cilia, and the basal body at the apical side of the cell. DRC, dynein regulatory complex.

shown that the proteins responsible for autosomal dominant polycystic kidney disease also localized to the renal primary cilia in healthy cells, and were involved in calcium conductance [6,7], while mutations discovered in genes encoding basal body and axonemal proteins in patients with the pleiotropic Bardet-Biedl syndrome phenotype highlighted the near ubiquitous critical roles of cilia across tissues [8]. It is now well-established that primary, non-motile, cilia play crucial roles in facilitating several signaling pathways, such as Wnt and Hedgehog signaling, and can also function as chemosensory, mechanosensory and photosensory

mediators [9–12]. For several years, only non-motile cilia were thought to be sensory organelles. However, recent observations have shown that motile cilia can also have chemosensory functions [13,14].

The ability of motile cilia to move or "beat" is due to the differences in microtubule composition when compared to immotile primary cilia (Figure 1D). All cilia are composed of a microtubule-core structure, the axoneme, and are encased in a membrane that is continuous with the plasma membrane. Ciliary microtubule formation is nucleated at the basal body, an anchoring structure at the base of the cilium that originates from the centrioles. Axoneme elongation occurs exclusively at the distal end, away from the base [15]. An intraflagellar transport (IFT) [16] system, consisting of several raft and motor proteins that move bi-directionally along the axoneme, is used to transport protein components from the cytoplasm to the ciliary tip. There are nine microtubule doublets connected by nexin links that circle the periphery of the cilium. Motile cilia have one microtubule doublet in the center (the central pair), generating a 9+2 arrangement that is not found in non-motile cilia (which have a 9+0 arrangement). Motile cilia also have two dynein arms, an inner and an outer (IDAs and ODAs, respectively), along the length of each microtubule doublet, which hydrolyze ATP to generate movement; these are also absent in non-motile cilia. A third category of cilia that shows characteristics intermediate between the two traditionally defined groups have been described at the embryonic node, a structure critical for establishing the first break of laterality in the body plan [17]. These "nodal cilia" have a 9+0 arrangement and dynein arms, facilitating a rotational movement [18], which is different to the planar beating of motile cilia in other tissues [19]. Individuals with PCD have defects in the function of both motile and also nodal cilia, suggesting that similar mechanisms must lead to the rotational and beating movements.

A major challenge in the management of PCD is obtaining early diagnosis. Patients affected with PCD present primarily with neonatal respiratory distress and chronic sinopulmonary disease that may be accompanied by organ laterality defects (heterotaxy) and, later, male infertility [20]. The sinopulmonary symptoms of PCD can be confused with other congenital diseases, such as cystic fibrosis (incidence of ~1:3500 births in populations of European descent) [21]. In the presence of clinical symptoms, a PCD diagnosis can be made through examination of the respiratory ciliary ultrastructure via electron microscopy, measurement of nasal nitric oxide levels, which are typically reduced in PCD patients [22–24], and functional testing of pattern and frequency of ciliary movement using high-speed video imaging [25]. However, the current diagnostic methods have significant drawbacks. PCD is genetically heterogeneous, with >30 causative genes reported (Table 1), accounting for ~65% of PCD cases [20]. Thus, for genetic testing to become a comprehensive diagnostic tool, additional genes need to be uncovered to account for PCD in the remaining ~35% of patients.

Here, we give an overview of PCD as a clinically distinct ciliopathy; we highlight the advantages and disadvantages of the currently used diagnostic methods; and we discuss how PCD gene discovery has improved our understanding of motile ciliary assembly.

PCD: a motile cilia disease

Motile cilia are found on cells lining the airways of the nasal cavity, middle ear, paranasal sinuses and the lower respiratory tract [26]. Consistent with these areas of function, PCD patients suffer from numerous respiratory problems. In addition, motile cilia are found on brain ventricular ependymal cells, and also line the fallopian tubes in women. Thus, although less common than other PCD symptoms, reduced female fertility [27], possibly increased risk of ectopic pregnancy [28,29] and hydrocephalus have been noted in some PCD patients [30–35]. Most PCD symptoms can evolve into serious health problems [36]. Bronchiectasis, or lung disease, is an almost universal outcome of chronic sinusitis in PCD patients and can require lung transplantation [37], and chronic middle ear infections can lead to transient or permanent hearing loss [38,39]. The presence of heterotaxy or complex changes to the arrangement of organs (12% of PCD patients) is also associated with complex cardiac malformations requiring surgery [37,40]. Early diagnosis of PCD and early treatment may help ameliorate some symptoms, and also prevent deterioration of lung function through regular physiotherapy and antibiotic treatment [41].

In some instances, PCD can co-segregate with X-linked mental retardation caused by mutations in *OFD1* (oral-facial-digital type 1), or with X-linked retinitis pigmentosa caused by *RPGR* depletion (Table 1) [42–45]. Furthermore, a PCD-like phenotype caused by reduced generation of multiple motile cilia (RGMC) has been described, which is characterized by sinopulmonary symptoms and fertility defects similar to those observed in PCD patients [46,47]. The residual motile cilia in RGMC caused by mutations in *CCNO* show a normal ciliary beat [46], while the few remaining motile cilia in patients with RGMC caused by *MCIDAS* mutations are immotile [47]. However, no *situs* defects have been observed in any patients with RGMC, thus suggesting that the function of nodal cilia is intact.

Table 1. Currently known causal primary ciliary dyskinesia (PCD) genes

Ultrastructural defect category	Gene	TEM defect/Function	% of PCD cases	References	Model organism	SD	NRD	RI	B	OM	Fertility affected	Ciliary beat characteristic
Outer dynein arm defect	*DNAH5*	ODA defects/Dynein arm component	~15-20%	[68–70]	NA	Yes	Yes	Yes	Yes	Yes	NA	Immotile cilia
	DNAI1	ODA defects/ODA component	~10%	[59,71,72]	*Chlamydomonas*	Yes	Yes	Yes	Yes	Yes	Yes	Immotile cilia
	NME8/ TXNDC3	ODA defects/ODA component	Rare	[61]	Sea urchin	Yes	NA	Yes	Yes	Yes	NA	Normal beat frequency
	DNAI2	ODA defects/ODA component	~2%	[73,104]	*Chlamydomonas*	Yes	NA	Yes	Yes	Yes	Yes	Immotile cilia
	DNAL1	ODA defects/ODA component	Rare	[74]	*Chlamydomonas*	Yes	NA	Yes	Yes	Yes	NA	Weak or no movement
	ARMC4	ODA defects/Docking and targeting of ODA components	~6%	[76,77]	Mouse, Zebrafish	Yes	Yes	Yes	Yes	Yes	NA	Reduced frequency or amplitude and immotility
	CCDC114	ODA defects/Docking of ODAs to microtubules	~4%	[78,79]	*Chlamydomonas*	Yes	Yes	Yes	Yes	Yes	No	Reduced frequency, dyskinetic or immotile
	CCDC151	ODA defects; ODA and IDA defects[1]/Assembly of ODAs and ODA docking complexes	NA	[80], [105]	Zebrafish, Mouse, Planaria	Yes	Yes	Yes	Yes	Yes	NA	Immotile cilia
	CCDC103	ODA and IDA defects/ Anchoring of dynein arms to microtubules	Rare	[65]	Zebrafish, *Chlamydomonas*	Yes	Yes	Yes	Yes	Yes	NA	Reduced amplitude, uncoordinated or immotile
	DNAAF3	ODA and IDA defects/ Cytoplasmic assembly of IDAs and ODAs	Rare	[60]	*Chlamydomonas*	Yes	Yes	Yes	Yes	Yes	NA	Immotile cilia
	DNAAF1/ LRRC50	ODA and IDA defects/ Cytoplasmic assembly of IDAs and ODAs	2-5%	[81,83]	Zebrafish, *Chlamydomonas*	Yes	NA	Yes	Yes	Yes	Yes*	Immotile cilia
	DNAAF2/ Ktu	ODA and IDA defects/ Cytoplasmic assembly of IDAs and ODAs	~2%	[63]	Medaka, *Chlamydomonas*	Yes	NA	Yes	Yes	Yes	Yes	Immotile cilia
Outer and inner dynein arm defects	*LRRC6*	ODA and IDA defects/ Cytoplasmic assembly of IDAs and ODAs, transcriptional regulation of dynein arm components	~3%	[84,106,107]	Zebrafish, *Drosophila*, *Chlamydomonas*	Yes	Yes	Yes	Yes	Yes	Yes*	Immotile cilia
	HEATR2	ODA and IDA defects/ Cytoplasmic assembly of IDAs and ODAs	Rare	[85]	*Chlamydomonas*	Yes	Yes	Yes	NA	Yes	Yes	Reduced motility
	ZMYND10	ODA and IDA defects/ Cytoplasmic assembly of IDAs and ODAs, transcriptional regulation of dynein arm components	~5%	[86,87]	Zebrafish, *Xenopus*	Yes	Yes	Yes	Yes	Yes	Yes*	Immotile cilia
	C21orf59	Loss of ODAs and IDAs/ Possible adaptor protein for ODA and IDA transport	~1%	[88]	*Chlamydomonas* Zebrafish, Planaria	Yes	Yes	Yes	Yes	Yes	NA	Immotile cilia
	SPAG1	ODA and IDA defects/ Cytoplasmic assembly of IDAs and ODAs	~3%	[89]	Zebrafish	Yes	Yes	Yes	Yes	Yes	NA	Stiff and immotile cilia

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	DYX1C1	ODA and IDA defects/ Cytoplasmic assembly of IDAs and ODAs	~3%	[66]	Mouse, Zebrafish	Yes	Yes	Yes	Yes	Yes	Yes	Immotile cilia
	CCDC40	Central microtubule pair, IDA and Nexin-DRC defects/ Assembly of Nexin-DRC complexes and role in IDA attachment	2-7%	[90,91]	Mouse, Zebrafish	Yes	Yes	Yes	Yes	Yes	Yes	Reduced amplitude or immotile cilia
Inner Dynein Arm/Dynein regulatory complex defects	*CCDC39*	Central microtubule pair, IDA and Nexin-DRC defects/ Assembly of Nexin-DRCs and role in IDA attachment	2-6%	[67,91,92]	Dog, Zebrafish	Yes	Yes	Yes	Yes	Yes	Yes	Reduced amplitude and defective beat regulation
	CCDC65	Defects in IDAs and nexin links and microtubule disorganization[2]/ Dynein regulatory complex component	Rare	[88,103]	*Chlamydomonas*, Zebrafish, Planaria	No	Yes	Yes	Yes	Yes	NA	Stiff, dyskinetic cilia
	DRC1/ CCDC164	Missing nexin links/Dynein regulatory complex component	Rare	[64]	*Chlamydomonas*	No	Yes	Yes	Yes	Yes	NA	Increased beat frequency with reduced amplitude of bending
	RSPH4A	Central microtubule pair defects[3]/Radial spoke protein	~2%	[57,95]	Zebrafish, Chlamydomonas, Mouse	No	Yes	Yes	Yes	Yes	Yes	Rotational, uncoordinated movement
	RSPH9	Central microtubule pair defects[3]/Radial spoke protein	~1%	[57,95]	Zebrafish, *Chlamydomonas*, Mouse	No	NA	Yes	Yes	Yes	Yes	Rotational movement
Central pair defects	*HYDIN*	Central microtubule pair composition[3]	Rare	[96]	Zebrafish, *Drosophila*	No	Yes	Yes	Yes	Yes	NA	Uncoordinated and reduced beat amplitude
	RSPH1	Central microtubule pair and radial spoke defects/Radial spoke protein	~2-3%	[57,58]	NA	No	Yes	Yes	Yes	Yes	Yes*	Reduced frequency
No ultrastructural defects	*DNAH11*	No ultrastructural defects/ Dynein heavy chain subunit	~6-7%	[62,97]	*Chlamydomonas*, Mouse	Yes	NA	Yes	Yes	Yes	No	Stiff, hyperkinetic beat
	OFD1[4]	NA; Centrosomal and primary cilia basal body protein; possibly involved in intraflagellar transport	NA	[42,108,109]	Mouse	NA	Yes	Yes	NA	NA	NA	Uncoordinated and disorganized beat
	RPGR[5]	Missing dynein arms, central pair and/or nexin links	NA	[43–45,110–117]	Dog, Zebrafish, Mouse	No	No	Yes	Yes	Yes	No	Mix of motile and immotile cilia
	CCNO[6]	NA; Mother centriole amplification and maturation during apical docking	NA	[46]	*Xenopus*	No	Yes	Yes	Yes	NA	Yes*	No obvious beating defects
Other	*MCIDAS*[6]	NA; Required for deuterosome-mediated acentriolar pathway; transcriptional activator of genes required for centriole formation	NA	[47,118–120]	*Xenopus*	No	Yes	Yes	Yes	Yes	Yes*	Immotile cilia
	DNAH8[7]	NA	NA	[121]	NA	NA	NA	NA	NA	NA	NA	NA

*Infertility or subfertility observed in female PCD patients as well. [1]Different transmission electron microscopy (TEM) defects were observed in different populations of PCD patients with mutations in *CCDC151*. [2]Microtubule disorganization was found in 5-15% of patients; Horani *et al.* [103] did not observe any ultrastructural defects in their cohort of patients with *CCDC65* mutations, suggesting that mutations in *CCDC65* cause mild structural defects. [3]Defects were seen in a small proportion of cilia; most ciliary ultrastructure appeared normal. [4]PCD is associated with X-linked mental retardation. [5]PCD is associated with X-linked retinitis pigmentosa. [6]CCNO and MCIDAS cause a PCD-like phenotype classified as reduced generation of multiple motile cilia (RGMC). [7]This is a preliminary finding of two null mutations in *DNAH8* in one patient. Functional studies of the mutation and also investigation of the ultrastructural and ciliary beat phenotypes have not been reported.

B, bronchiectasis; DRC, dynein regulatory complex; IDA, inner dynein arm; ODA, outer dynein arm; OM, otitis media; NA, not available; NRD, neonatal respiratory distress; RI, respiratory infection; SD, *situs* defect;

Diagnosing PCD

The similarity of the respiratory symptoms of PCD with common childhood problems, and the lack of awareness of this rare disorder (prevalence of ~1 in 10,000) [48] often impede early diagnosis. A recent study reported that the median age at diagnosis was ~5.3 years [49]. By this time, there can already be considerable damage to the lung tissue in affected children if untreated [37,50]. There are several diagnostic methods currently used. Transmission electron microscopy (TEM) of cilia from nasal brushings can identify defects in the ciliary structure, such as defective ODAs or disorganized microtubules [51]. However, since ~30% of PCD patients do not show defects in ciliary structure [52,53], this method cannot be used as an exclusive means to diagnose the disease. Nasal brushings can be analyzed directly or can also be maintained in cell culture to assess ciliary movement by high-speed video microscopy. However, such analyses require specialized personnel and laboratories (as does TEM) to look at cilia structure, and are not readily accessible to all patients. In addition, ciliary dysfunction can also be acquired from infections that result in inflammation, known as secondary ciliary dyskinesia (SCD) [54–56]. Therefore, it is important to distinguish whether abnormalities in ciliary structure or function are due to PCD or SCD. A relatively simple screening method is measurement of nitric oxide (NO) levels in the nasal passage, which are reduced typically in PCD patients compared to healthy individuals. However, other conditions, such as cystic fibrosis and severe sinusitis, can also cause abnormally low levels of NO, and the range of nasal NO values that distinguish between PCD and these other conditions are yet to be standardized [24]. In addition, some PCD-causing mutations may not result in nasal NO levels low enough to offer a conclusive diagnosis [57,58]. Therefore, this test can provide a first pass screen for PCD in children >5 years of age, rather than a diagnosis of the disorder. Finally, DNA sequencing to identify causal mutations is becoming the benchmark for diagnosis of several genetic disorders. The applicability of clinical genetic testing for PCD diagnosis is currently limited by the fact that PCD is highly genetically heterogeneous.

Genetics of PCD

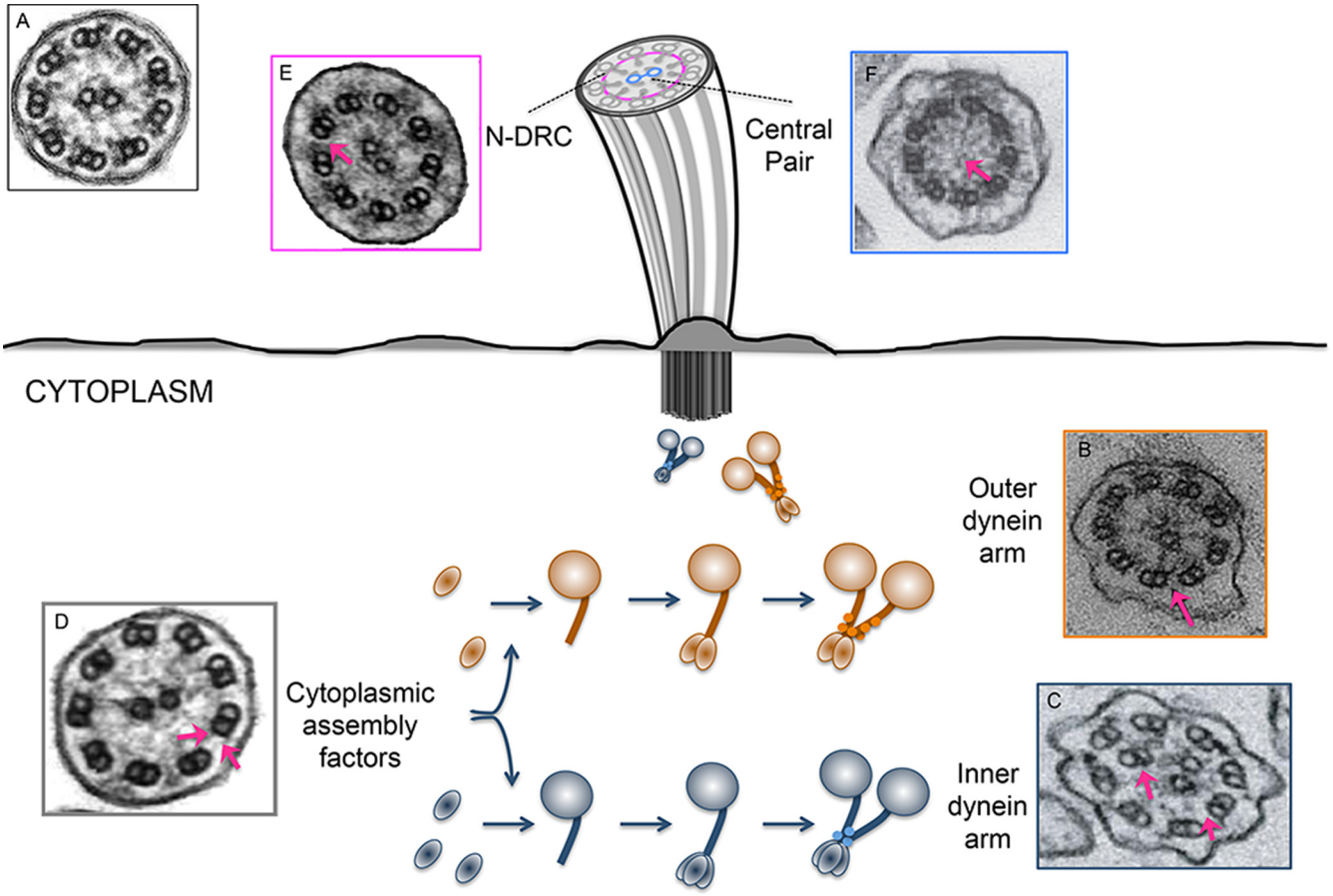
PCD is inherited primarily in an autosomal recessive manner. The first gene shown to be mutated in individuals with PCD was *DNAI1*, encoding an axonemal dynein intermediate chain [59]. Pennarun *et al.* used a candidate gene approach to screen a PCD cohort for *DNAI1* mutations because a *Chlamydomonas reinhardtii* mutant bearing mutations in the orthologous gene showed flagella with a TEM defect affecting ODAs similar to that observed in some PCD patients [59]. PCD-related phenotypes uncovered initially and studied in model organisms, such as *Chlamydomonas*, medaka, zebrafish, sea urchins, *Xenopus*, mice and even dogs have led to the sequencing and identification of several causal PCD genes [60-67] (Table 1). Not surprisingly, many of these genes are structural components of the axoneme, involved in the composition of the ODAs or their attachment to microtubules (e.g. *DNAH5*, *TXNDC3*, *DNAI2*, *DNAL1*, *ARMC4*, *CCDC114*, and *CCDC151*). Mutations in these genes result typically in the complete or partial absence of the ODAs as observed by TEM (Figure 2), and corroborated by the complete or partial absence of ODA markers, such as DNAH5, DNAI2 and DNAH9 in affected axonemes [59,61,65,68–80].

Molecular mechanisms of motile cilia assembly

In addition to aiding diagnosis, gene discovery has advanced our understanding of basic cellular processes underscoring ciliary biogenesis and function. This is exemplified by the discovery of *Ktu*/*DNAAF2*, a locus that, when mutated, causes a loss of both the ODAs and IDAs, but is not detectable in the ciliary fraction of cell extracts [63]. Subsequent experiments showed that Ktu is a cytoplasmic protein involved in the pre-assembly of both ODAs and IDAs before they are transported into the cilium [63] (Figure 2). This discovery introduced a novel paradigm to explain motile cilia formation; prior to the implication of *Ktu* in PCD, the existing repertoire of causal genes were structural components of the cilium and their site of assembly during ciliogenesis was not known. Following *Ktu*, nine other cytoplasmic proteins have been linked causally to PCD (*CCDC103*, *DNAAF3*, *DNAAF1*, *LRRC6*, *HEATR2*, *ZMYND10*, *C21orf59*, *SPAG1*, and *DYX1C1*; Table 1). To date, mutations in all the cytoplasmic dynein assembly factors associated with PCD appear to cause defects in both the IDAs and ODAs according to TEM (Figure 2). This has also been shown by the absence, reduction or mislocalization of ODA and IDA markers, as visualized by the immunofluorescent evaluation of respiratory epithelial cells [60,63,66,81-89].

The identification of genes mutated in PCD cohorts displaying concomitant IDA and central pair defects have elucidated the composition and roles of the nexin-dynein regulatory complex (N-DRC) in ciliary formation (Figure 2). Isolated IDA defects have not been reported in PCD; rather, complete or partial absence of IDAs have thus far been associated with disorganization of peripheral microtubule doublets (in some instances, the central pair of microtubules may also be absent, displaced, or supernumerary). These defects are caused by mutations in proteins involved in the attachment of the IDAs to the microtubules, and in the assembly of the N-DRC (*CCDC40*, *CCDC39*, and *CCDC65*; Table 1) [64,67,88,90-93]. Therefore the IDA

Figure 2. Illustration of ultrastructural ciliary defects



These ultrastructural ciliary defects are characteristic of mutations in genes involved at different steps of cilia assembly and function, including the assembly of the inner and outer dynein arms by cytoplasmic assembly factors. Transmission electron microscopy (TEM) images are shown of a control sample (A, reproduced with permission from Elsevier) and patients with mutations in the following: B. *DNAH5*, showing defective outer dynein arms (ODAs) (reproduced with permission from Elsevier), C. *CCDC40*, showing defective inner dynein arms (IDAs) and nexin links (reproduced with permission from Nature Publishing Group), D. *LRRC50*, showing missing IDAs and ODAs (reproduced with permission from Elsevier), E. *CCDC164*, showing defects in the nexin- dynein regulatory complex (N-DRC) (reproduced with permission from Nature Publishing Group), and F. *RSPH1*, showing missing central pair of microtubules (reproduced with permission from Elsevier).

defects are also accompanied by defects in the N-DRC, as visualized by the absence or reduction of N-DRC marker proteins (such as GAS11). This sub-group of PCD proteins is unique because, when rendered dysfunctional, they leave only ODAs unaffected; as a result, the force-generating structures are intact, but lack the proper beat regulation likely conferred by the IDAs, the N-DRC and central pair microtubules [94].

Correlations between genotype, ciliary defect and clinical phenotype

Mutations in a subset of proteins, primarily the components of the radial spokes (*RSPH1, RSPH9, RSPH4a,* and *HYDIN*; Table 1), give rise to abnormalities in the central pair without affecting the ODAs or IDAs (Figure 2). In contrast to individuals displaying the gamut of classic PCD symptoms that are caused by IDA and N-DRC defects, accompanied by central pair aberrations, patients carrying mutations that affect only the central pair of microtubules do not present with laterality defects [57,58,95,96]. Notably, accumulating clinical data from this PCD subcohort show that the movement of nasal epithelial cilia carrying a defective central pair of microtubules is circular [58,95,96], distinctly different from the planar beating of healthy cilia. This movement is reminiscent of that of nodal cilia, which do not have a central pair of microtubules [18]. Thus, a credible explanation for an absence of left-right asymmetry defects in patients harboring central pair abnormalities is that nodal cilia exhibit normal movement and beat frequency.

While ciliary ultrastructural defects are a hallmark of PCD in the majority of patients, a significant fraction of individuals with clinical PCD symptoms present with normal ciliary structure (~30%), but abnormal function. Of this subset, conservative estimates indicate that 22% of patients have biallelic mutations in *DNAH11*, although one study that also considers the contribution of possibly pathogenic rare alleles estimates that as many as 70% of patients have candidate causal biallelic variants in this locus [52,53] (Table 1). It is intriguing that this gene codes for a component of the dynein heavy chain, although no adverse effects on ciliary structure, both via TEM or immunofluorescence analysis, have been detected in patient respiratory epithelia. Analysis of ciliary movement from individuals harboring *DNAH11* mutation, using high-speed video microscopy, shows a non-flexible and hyperkinetic pattern that is different to the nearly immotile cilia seen in patients with *DNAH5* or *DNAI1* mutations (abnormal ODA; Figure 2) [97], and more closely resembles that of disrupted N-DRC. The normal ultrastructure of cilia in patients with *DNAH11* mutations was a key discovery in PCD biology as it indicated that not all instances of PCD manifested in ciliary structural defects. It also highlighted the need for several diagnostic avenues, such as high-speed microscopy to visualize cilia movement and identification of genetic mutations/lesions in addition to TEM, in providing a clinical diagnosis.

Despite the differences in the ultrastructural phenotypes at the organellar level and the resulting ciliary beat differences in *DNAH11* versus *DNAH5* or *DNAI1* mutation-bearing individuals, loss of function mutations in any of these loci result in the classic PCD phenotype [52,53,97]. One notable exception may be the observation by Schwabe *et al*. [97] that fertility does not seem to be affected in the presence of *DNAH11* mutations. Since normal fertility was noted only in one male from one affected family, caution is warranted in concluding that this is a general characteristic of *DNAH11* mutations. However, it is possible that effects of ciliary gene mutations differ between motile cilia in respiratory epithelia and sperm flagella. This notion is supported by the reported differences in axoneme composition, specifically the dynein heavy chain subunits, between these two types of cilia [98]. In addition, mutations in another gene (*CCDC114*) have also been shown to cause PCD without an overt infertility phenotype [78] (Table 1). Together, these observations suggest that the ultrastructural differences resulting from different PCD mutations result in similar end phenotypes in patients, with the noted exceptions of *situs* defects and fertility.

Although an individual's clinical PCD symptoms may not be sufficient to elucidate which group of genes may harbor mutations, the TEM and immunofluorescence analysis of cilia structure can be helpful in narrowing the pool of candidates. For example, defects in both ODAs and IDAs are indicative of underlying mutations in cytoplasmic dynein assembly factors; and the absence of any ultrastructural defect in the presence of PCD-like symptoms suggests the increased likelihood of mutations in *DNAH11* or other genes that have been associated with subtle or unappreciable TEM defects, such as *CCDC65*, *CCDC164* or *HYDIN*. Correlations of axonemal ultrastructure and functional output data with gene groups stratified by function have been used to direct sequencing efforts and facilitate diagnosis [57,69,76,84].

The majority of mutations reported in PCD patients are null alleles (frameshift or nonsense). There are few instances of mutations that have been tested and characterized as hypomorphic. Moore *et al*. [87] characterized a missense mutation in *ZMYND1* (V16G) as a hypomorph in cell-based assays and in *Drosophila* models. However, even though cilia from patients homozygous for V16G retained some motility, the clinical phenotypes of these patients were similar to those of PCD individuals harboring null alleles. Similarly, Knowles *et al*. [79] reported leaky expression of the full-length mRNA in a patient with two splicing mutations in *CCDC114*, suggesting that the alleles had residual function, although the patient's phenotype was indistinguishable from that of patients with *DNAI1* or *DNAH5* mutations. Panizzi *et al*. [65] determined, through functional studies in zebrafish, that a missense mutation in *CCDC103* (H154P) identified in PCD patients was a hypomorph. Even so, the clinical data from the *CCDC103* mutation-bearing patients were consistent with full PCD diagnostic criteria. Together, these observations suggest that the tolerance threshold for motile ciliary perturbations is low, and that correlations between PCD protein function and disease phenotypes may be imperfect.

A study comparing different types of mutations in *DNAH5* concluded that two mutations causing premature termination of translation resulted in a complete loss of the ODAs in those patients, while cilia with a splicing mutation in *DNAH5* had a partial absence of ODAs [99]. However, how these ultrastructural defects correlated with the clinical symptoms of the patients carrying these mutations was not provided. Therefore, more studies that compare the severity of mutations through functional tests and the clinical phenotypes of

patients are needed to understand the contributions of mild versus severe mutations to the PCD phenotype.

Concluding remarks

Hundreds of genes have been implicated in motile cilia structure and function [100], thus representing a sizeable repertoire of candidate genes that could be involved in PCD etiology. Gene discovery in the PCD field has accelerated, as whole exome and whole genome sequencing (WES/WGS) have become increasingly affordable and widely used. Over half of the causal PCD genes (16) have been uncovered within the last 4 years, compared to nine that were implicated in the 11 years after the association of *DNAI1* with the disease (Table 1). As more patient cohorts are sequenced, we will likely discover new candidate genes to account for the 35% of the remaining patient population with an unresolved molecular genetic diagnosis.

Despite the genetic heterogeneity underlying PCD, the patient phenotypes reported are largely similar, with few examples of gene- or mutation-specific differences (Table 1). The exceptions include an absence of *situs* defects and infertility [57,78,95,96]. In a recent study, Knowles *et al*. [58] observed that their cohort of patients with mutations in *RSPH1* had mild PCD phenotypes, which included a lower incidence of neonatal respiratory distress, later onset of respiratory problems, better lung function and higher nasal NO levels when compared to patients with mutations in *DNAH11*. In general, the phenotype of patients with *RSPH1* mutations is milder, compared to PCD patients with mutations that cause ultrastructural defects affecting the inner or outer dynein arms [37,58]. An earlier study, however, did not find significantly milder phenotypes in patients with *RSPH1* mutations [57]. This discrepancy might be due to differences in clinical ascertainment. Nevertheless, the *RSPH1* observation is notable; it is among the few reports of a possible genotype-phenotype correlation in PCD and it highlights the importance of having comprehensive and quantitative clinical data to enable comparisons between groups of patients.

In comparison to the primary ciliopathies, the clinical homogeneity of PCD is striking. We note with interest that a significant proportion of variants associated causally with PCD genes are null mutations. Is this observation due to an ascertainment bias in which severe, syndromic patients undergo molecular analysis? PCD cohorts in genetic studies are diagnosed typically based on clinical symptoms and cilia structure/function analysis. However, this approach can be problematic, as many of these symptoms can be due to other causes. It is possible that residual activity in many of the PCD-causing genes is sufficient to spare the organism from pathology, or that the hypomorph of PCD might be a completely different disorder, a paradigm well-described for the primary ciliopathies [101].

Consistent with the lack of clinical variability in PCD, and despite the accumulating evidence for oligogenic phenomena contributing to the phenotypic variability observed in the primary ciliopathies, genetic interactions in PCD have been essentially unexplored. One possibility is that mutations in PCD genes have an "all or none" effect on motile ciliary function, such that an increase in the number of mutations in ciliary genes is not expected to exacerbate the clinical phenotype. The fact that mutations in most causal PCD genes produce the classic PCD phenotype argues in favor of this possibility. Alternatively, the concept of ciliary gene mutational load cannot be excluded as a contributor to the phenotype in some patients. The exacerbation of the phenotypic severity or the manifestation of certain endophenotypes by the genetic interaction of two or more ciliary gene mutations is a well-established phenomenon in other ciliopathies [101,102]. To appreciate what role, if any, genetic interaction plays in PCD, we need a detailed understanding of genotype-phenotype correlations both within PCD pedigrees with multiple affected individuals, and also among unrelated individuals who harbor mutations at the same locus. Additionally, unbiased approaches, such as WES and WGS, are required to identify the primary "driver" of PCD mutations as well as potential phenotypic modifier mutations.

Looking forward, a key goal is to identify more causal PCD genes that can improve the diagnostic power when combined with other approaches, such as TEM and ciliary function analysis. Moreover, the continued standardization and improved quantitative means of reporting clinical phenotyping will aid our ability to make informed correlations between mutational burden and phenotype, both within and across families. We anticipate that the continued intersection of robust *in vivo* and *in vitro* tools with comprehensive genetic and clinical data will ultimately decrease time to diagnosis and improve clinical outcomes for this life-threatening disorder.

Abbreviations

IDA, inner dynein arm; N-DRC, nexin-dynein regulatory complex; NO, nitric oxide; ODA, outer dynein arm; PCD, primary ciliary dyskinesia; RGMC, reduced generation of multiple motile cilia; SCD, secondary ciliary dyskinesia;

TEM, transmission electron microscopy; WES, whole exome sequencing; WGS, whole genome sequencing.

Disclosures

The authors declare that they have no disclosures.

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