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INVITED REVIEW

Motile and non-motile cilia in human pathology: from function to phenotypes

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Abstract

Ciliopathies are inherited human disorders caused by both motile and non-motile cilia dysfunction that form an important and rapidly expanding disease category. Ciliopathies are complex conditions to diagnose, being multisystem disorders characterized by extensive genetic heterogeneity and clinical variability with high levels of lethality. There is marked phenotypic overlap among distinct ciliopathy syndromes that presents a major challenge for their recognition, diagnosis, and clinical management, in addition to posing an on-going task to develop the most appropriate family counselling. The impact of next-generation sequencing and high-throughput technologies in the last decade has significantly improved our understanding of the biological basis of ciliopathy disorders, enhancing our ability to determine the possible reasons for the extensive overlap in their symptoms and genetic aetiologies. Here, we review the diverse functions of cilia in human health and disease and discuss a growing shift away from the classical clinical definitions of ciliopathy syndromes to a more functional categorization. This approach arises from our improved understanding of this unique organelle, revealed through new genetic and cell biological insights into the discrete functioning of subcompartments of the cilium (basal body, transition zone, intraflagellar transport, motility). Mutations affecting these distinct ciliary protein modules can confer different genetic diseases and new clinical classifications are possible to define, according to the nature and extent of organ involvement.

Keywords: cilia; ciliopathies; signalling; ciliogenesis; kidney cystic diseases; Joubert syndrome; skeletal dysplasia; Meckel syndrome; Bardet–Biedl syndrome; oral-facial-digital syndrome

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Introduction

Cilia are highly conserved organelles projecting from the surface of virtually every cell type in the vertebrate body that are found ubiquitously across species from nematodes to ancient protozoa. These complex and dynamic structures are broadly divided into motile and non-motile subtypes that share a 25 μm micrometre diameter cytoskeletal scaffold, the axoneme, composed of hundreds of proteins [1,2]. The axoneme contains nine peripheral microtubule doublets, consisting of A and B tubules, either surrounding a central pair of microtubules (9 + 2 pattern) or lacking the central pair (9 + 0 pattern). Motile 9 + 2 cilia exist as multiple cilia (multicilia), whilst 9 + 0 motile and non-motile cilia exist as single monocilia on the cell surface. Almost all human cells possess a single non-motile (primary or sensory) cilium, whereas multicilia are generated by specialized cells, and sperm tail (flagella) motility also employs a highly conserved axonemal structure.

During cilia formation (ciliogenesis), the axoneme nucleates from a centriole-derived basal body that docks at the plasma membrane, extending out a microtubule bundle contained within a specialized extension of the plasma membrane that harbours selected signalling molecules and ion channels [3]. Lacking machinery for protein synthesis, proteins of the ciliary compartment synthesized in the Golgi apparatus are imported through a ciliary 'gate' located towards the cilia base. This consists of the basal body with transition fibres and a transition zone area above, which directs entry of cargos for their subsequent transport along the length of the cilia [4]. Intraflagellar transport of cargos, IFT, occurs

in a bidirectional manner with the anterograde and retrograde direction of traffic mediated, respectively, by kinesin-2 and cytoplasmic dynein-2 motors attached to multisubunit protein complexes called IFT particles (Figure 1) [5,6]. The functional importance of cilia has emerged in recent years with the recognition of their central role in normal human development and also in disease. 'Ciliopathies' are complex multisystem human disorders of cilia with involvement of all the major organs including the kidney, brain, eye, airways, and limbs; they contribute subtypes to many common diseases such as retinal dystrophy and kidney disease [7–10]. This expanding disease category includes many syndromes such as primary ciliary dyskinesia (PCD), autosomal-dominant and -recessive polycystic kidney diseases (ADPKD, ARPKD), nephronophthisis (NPHP), Leber congenital amaurosis (LCA), and Bardet–Biedl (BBS), Senior–Løken (SLS), Joubert (JBTS), Jeune (or asphyxiating thoracic dystrophy, JATD), short rib polydactyly (SRPS), Meckel–Gruber (MKS) and oral-facial-digital (OFD) syndromes. These inherited disorders are individually rare, but collectively may affect up to 1 in 2000 people [11]. Ciliopathies share common clinical features and there is considerable genetic and phenotypic overlap as well as genetic heterogeneity [12].

Many reviews are available on the structure and functioning of cilia. Here, we present a global overview of the eclectic functions of cilia in different organs and tissues, referring to specialized reviews for further details; moreover, we summarize the spectrum of cilia-related phenotypes in relation to their genetic determinants, and discuss the increasing need to change the way of looking at cilia-related diseases, shifting from the classical definition of ciliopathy syndromes (and related acronyms) to a more descriptive approach based on the type and extent of organ involvement.

Motile ciliopathies

Motile ciliopathies are characterized by dysfunction of tissues, organs, and gametes that bear the specialized ciliary and flagella machinery for generating fluid flow or movement within fluids. Failure of these mechanisms compromises mucus clearance, causing chronic airway diseases which are associated with defects of laterality, fertility, and brain development. The hallmark disease of motile cilia is PCD [13]. About half of affected individuals have laterality defects, most commonly *situs inversus totalis* (mirror image reversal of the internal organs, Kartagener syndrome). A proportion of cases have more complex laterality defects dominated by left isomerism with congenital heart disease [14,15]. Oligocilia or reduced generation of multiple motile cilia (RGMC) is a subtype of PCD presenting the same disease spectrum but with a distinct aetiology: PCD and Kartagener syndrome result from structural and assembly defects of ciliary components, whilst RGMC arises from defects in the multiciliogenesis programme and is not associated with laterality defects [16,17].

Structure and function of motile cilia

Motile cilia line the epithelial surfaces of the upper and lower respiratory tracts and middle ear, the ventricles of the central nervous system, and the Fallopian tubes. They show variable length (e.g. brain ependymal cilia are longer and beat faster than lung cilia [18]), and axonemal arrangement (9 + 2 in respiratory and Fallopian tube cilia and sperm flagella; 9 + 0 in nodal cilia) [19–21]. Many microtubule-associated multisubunit structures attach with regular periodicity along the axoneme, creating a stable membrane-bound rod that supports and regulates dynein motor-based motility and waveform (Figure 2). Notably, only motile cilia and sperm flagella contain dynein motor proteins that power axonemal beating through ATP hydrolysis [22]. The outer and inner dynein motors are at 96 nm periodicity along the peripheral A tubule, projecting between the peripheral doublets, and two 'molecular ruler' proteins are required to maintain this periodicity [23]. Nexin–dynein regulatory complexes (N-DRCs) link between adjacent peripheral doublets to regulate dynein activity and facilitate inner dynein arm attachment, thereby governing axonemal waveform [24,25]. Radial spoke complexes in close proximity to the inner dynein arms project inwards to the central microtubule apparatus, providing a radial scaffold between the central apparatus and the peripheral microtubules for mechanochemical signal transduction that governs the ciliary beat and waveform [26]. Axoneme structure can vary; for example, the dynein arms differ in composition at the cilia base and tip [27].

The motor and signalling functions of this complex superstructure maintain a uniquely coordinated self-propagating beat [28]. Motile 9 + 0 monocilia of the embryonic left–right organiser beat unidirectionally [21], whilst motile 9 + 2 multicilia form a lawn of 200–300 cilia per cell, creating a coordinated metachronal wave moving at 1000 beats per minute to move fluids. Sperm flagella, though broadly similar in their 9 + 2 arrangement [29], differ in their detailed structure, such as the distribution of dynein arms along the axoneme [27,30]. The sigmoidal, symmetric three-dimensional movement of sperm flagella is also completely distinct from the cilia's planar asymmetric effective and recovery strokes [28,30].

Motile and non-motile cilia in left–right axis determination

Both motile and non-motile primary cilia are essential for establishing correct left–right patterning and the asymmetric positioning of internal organs, a physiological condition termed '*situs solitus*'. Both motile

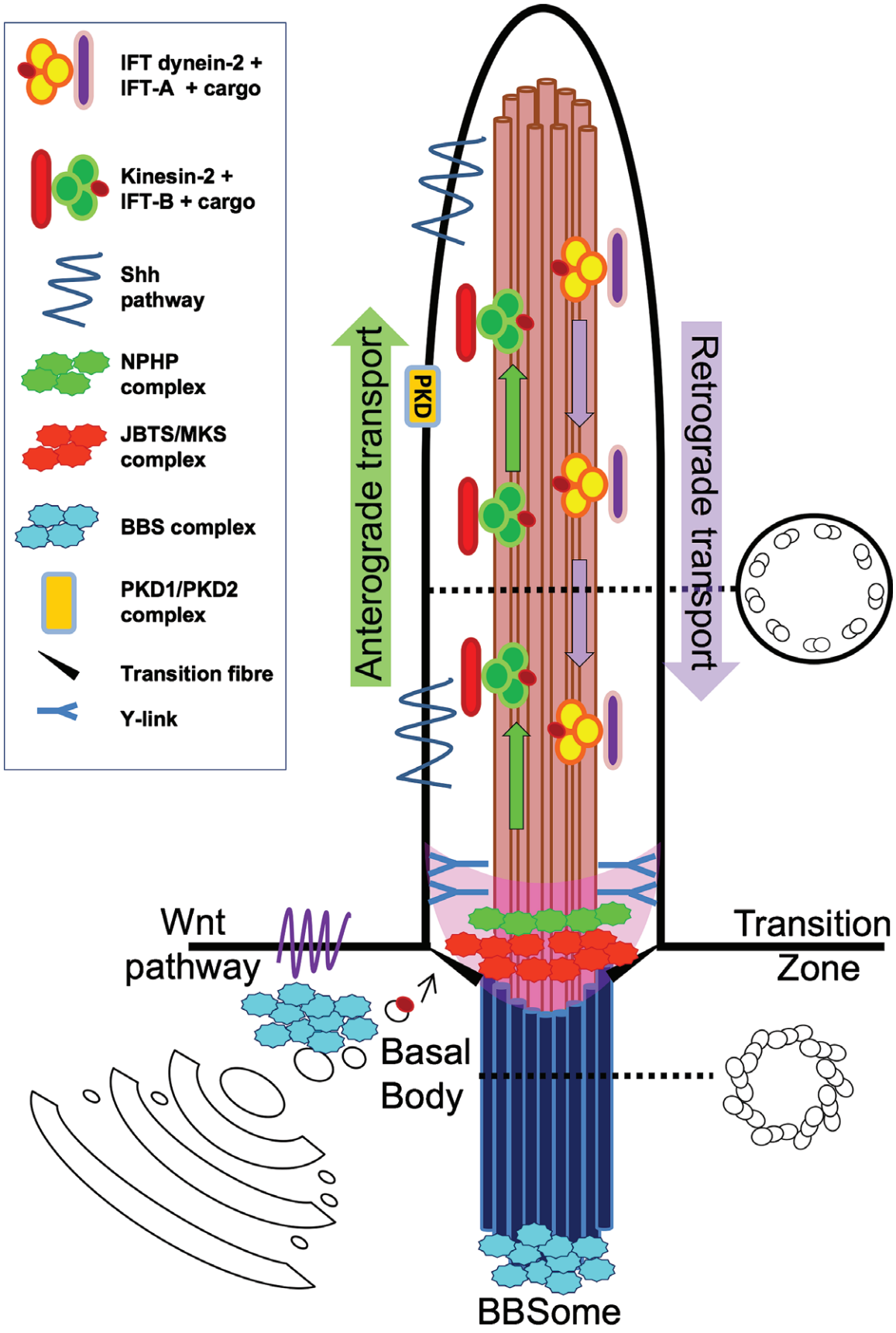

Figure 1. Schematic structure of the primary cilium depicting the main subciliary compartments: the transition zone (pink), containing proteins of the JBTS/MKS complex and the NPH complex; IFT subcomplexes IFT-A and IFT-B (mediating retrograde and anterograde transport, respectively); the BBSome; and the PKD1/PKD2 membrane complex. The relationship of the main developmental pathways Shh and Wnt with the primary cilium is also indicated. Figure adapted from ref 142.

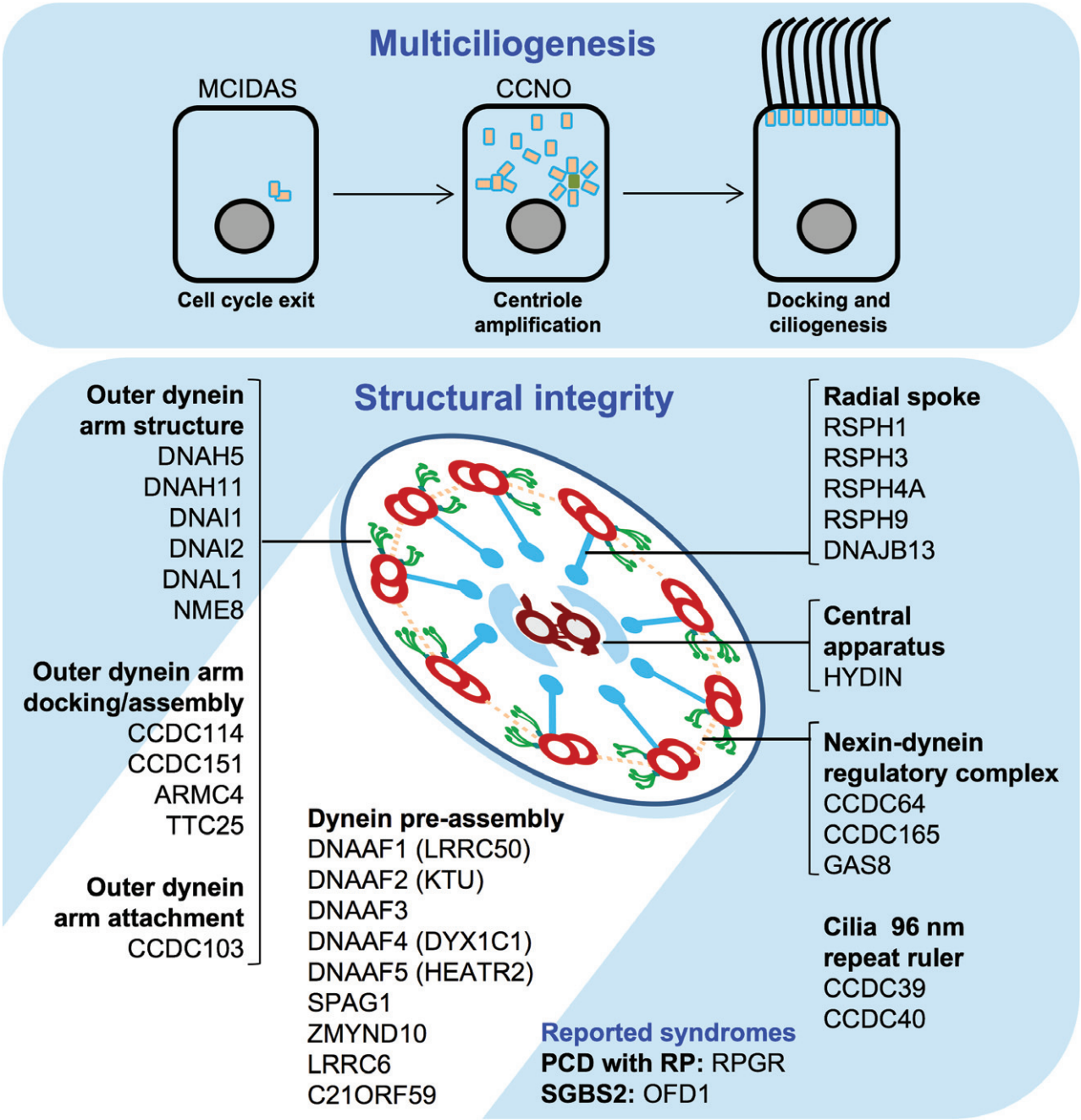

Figure 2. Structure of motile cilia and role of mutant proteins. Motile ciliopathies are caused by mutations in (top panel) components of the ciliogenesis pathway or (bottom panel) structural and attachment proteins of the axoneme dynein 'arm' motors (green), the dynein arm docking complex, the nexin–dynein regulatory complex (dotted lines), the central apparatus (brown), the radial spokes (blue), as well as molecular ruler proteins and cytoplasmic dynein arm assembly factors. Reported syndromes are: PCD associated with retinitis pigmentosa (RP) and Simpson–Golabi–Behmel syndrome, type 2 (SGBS2).

and non-motile ciliopathies can manifest with left–right axis patterning defects. The left–right organiser, part of the embryonic node, appearing early during embryonic development, possesses two types of 9+0 cilia: centrally placed singleton motile cilia and peripherally placed primary immotile cilia. Fluid flow across the embryonic node is a crucial first step in early embryogenesis, marking the earliest point of breaking of bilateral embryo symmetry [21]. Motile node cilia have dynein motors but their lack of a central pair creates a clockwise motion and they rotate at an average 600 rpm to generate a leftward fluid flow in the extracellular space. The peripheral immotile cilia sense the flow and respond, transmitting signals to the lateral plate mesoderm, which activates an asymmetric gene expression cascade; the most important downstream effector gene is *Nodal*, a member of the TGFβ growth factor family, which induces the asymmetric transcription of downstream genes, activating a self-propagating signalling cascade that establishes LR laterality and the vertebrate body plan [21,31,32]. Left–right patterning is also governed by FGF signalling, which controls the Shh pathway and ciliary length during embryonic development [33,34].

Pathogenesis of motile ciliopathies

Motile cilia in the airways have a prominent role in host defence against infection from inhaled microorganisms and other particles. Airway mucociliary clearance forms an important 'self-cleaning' mechanism by the mucociliary escalator, whereby collaborating mucus-producing epithelial goblet cells and multiciliated cells move mucus containing trapped pathogens and pollutants either up or down to the throat, to be ingested or expelled [35,36]. In the brain, 'ependymal flow' of cerebrospinal fluid (CSF) is generated by multicilia lining the ventricles, which moves signalling molecules through the central nervous system and maintains structure [37,38]. In females, the multiciliated Fallopian tube epithelia assist in the transport of eggs to the uterus, whilst the male gametes are propelled towards the uterus by sperm flagella motility.

In motile cilia diseases, failure of mucociliary clearance is often evident from birth with neonatal respiratory distress. Throughout life, there is progressive accumulation of mucus and pathogens causing obstruction and infections in the sinuses, ears, and lungs [13]. Ultimately the recurring lung infections associated with damage to the lungs can lead to irreversible bronchiectasis and permanent loss of lung function. In cases where laterality is affected, there may be cardiac disease associated with *situs ambiguous* that can be associated with severe congenital heart defects requiring surgery and transplant, and other features of isomerism such as polysplenia and asplenia [14,15]. There is also subfertility in affected males and females, due to reduced and immotile sperm and poor movement along the Fallopian tubes of eggs towards the uterus, with ectopic pregnancy reported.

The central nervous system can also be affected, as brain malformations and hydrocephalus can arise from dysmotility of ependymal cilia. Whilst common in PCD mouse models, hydrocephalus is rare in human PCD, likely due to species differences in the size and structure of the ventricular system. In *DNAH5*-mutant PCD mice, loss of ciliary ependymal CSF flow through the narrow cerebral aqueduct connecting the third and fourth ventricles is thought to contribute to aqueduct closure and consequent triventricular hydrocephalus in the early postnatal brain development period, while the larger aqueducts of the human brain may not be so susceptible to ventricle obstruction [37]. Notably, hydrocephalus is significantly more frequent in human PCD caused by multiciliogenesis defects, where cilia numbers are reduced (RGMC), than in PCD, where the multicilia, even if static, are still present [16,17,39]. The reasons for this are not known, but could be connected to better aqueduct structure maintenance if there are still cilia present, or because the cilia are not always fully static in PCD depending on the underlying mutations. In addition to blocked CSF circulation, hydrocephalus may in fact be initiated by ciliated epithelial cells of the choroid plexus (CPECs), the secretory cell region within each ventricle that produces CSF [40]. CPEC cilia are poorly characterized but are reported as transiently motile during the perinatal period; however, rather than or additional to motility functions, it may be that defective sensory functions of non-motile cilia also present on CPECs contribute to hydrocephalus through defective ion transport, which is proposed to govern CSF production [40,41].

Motile cilia diseases are genetically heterogeneous, caused by mutations in more than 30 genes affecting dynein motors and other structural components or dynein arm assembly of the multicilia in PCD [13,42–44], whilst multiciliogenesis gene mutations cause RGMC [16,17] (Figure 2). A biological stratification of the motile ciliopathies is starting to emerge since certain features of PCD do not manifest with selected gene mutations. For example, loss of central microtubular pair function caused by mutations in genes such as the *HYDIN* and *RSPH* genes is associated with a lack of laterality defects, since nodal monocilia do not require the central apparatus for motility [13,45]. Interestingly, mutations affecting the radial spokes and nexin–dynein regulatory complexes (N-DRCs) do not cause laterality defects, even though radial spoke and N-DRC genes are expressed at the embryonic node [46,47]. The multiciliogenesis gene defects associated with RGMC affect respiratory and brain cilia motility but apparently not sperm or nodal cilia, Since their mutation does not cause male infertility or laterality defects, though severe cases of hydrocephalus are seen [16,17,39]. It is also apparent that the function of a growing number of ciliary proteins present in the lungs may be provided by other proteins in the sperm, thus causing PCD without male infertility [48].

Non-motile ciliopathies

Non-motile or sensory ciliary disorders represent an expanding group of highly heterogeneous inherited disorders caused by defects in assembly or functioning of the 9 + 0 primary cilium. A wide phenotypic variability is notable amongst primary ciliopathies compared with motile ciliopathies, and extensive genetic and clinical overlaps among distinct conditions reflect their underlying molecular complexity. Indeed, non-motile cilia are much more ubiquitous in the body, functioning as key sensors of extracellular molecules that regulate numerous intracellular signal transduction cascades. Through signalling, primary cilia activate a wide range of responses including modulation of key developmental pathways, control of cell polarity in epithelial tissues, transduction of sensory stimuli, and regulation of stem cell proliferation and maintenance. Bioactive extracellular vesicles (EVs) have also been identified at cilia tips [49,50] and exosomes bearing ciliary membrane proteins have been isolated from the liver and urine [51,52], so cilia have the potential to release vesicles considered to have signalling roles in addition to their capacity to receive sensory signals [52]. Here, we present an overview of the diverse functions of primary cilia as they

relate to different organs of the body and distinct ciliopathy phenotypes. A schematic diagram of the primary cilium with the different subcompartments referred to below is shown in Figure 1.

Functions of primary cilia in embryonic and adult life

Kidneys

Primary cilia protrude from the apical surface of epithelial cells lining the nephron tubule and collecting ducts, in contact with urine flow. In the adult kidney, cilia act as sensory antennae that respond to modifications of urine flow, composition, and osmolality by modulating important intracellular signalling pathways [10,53,54]. For instance, polycystin-1 and −2, the two proteins mutated in ADPKD, were found to regulate a urine-flow dependent, calcium-mediated intracellular response able to influence several signalling pathways, such as G-protein signalling, mTOR, Wnt, and even Sonic Hedgehog (Shh) [55]. Similarly, mutations in ciliary proteins such as inversin or nephrocystin-3, which cause infantile and juvenile nephronophthisis (NPH), alter the balance between canonical and non-canonical Wnt pathways that is essential to control the correct polarity of epithelial tubular cells, thus explaining the pathogenesis of cyst formation [56].

Cilia defects in the kidneys typically lead to the development of cystic kidney diseases. Cysts may form at any age from prenatal to adult life; can vary widely in number, size, and distribution; and in some cases, are associated with progressive interstitial fibrosis, defining a wide spectrum of renal ciliopathies. While the pathomechanisms of cyst formation have largely been elucidated [57,58], how ciliary dysfunction leads to excessive interstitial fibrosis still remains a matter of debate. Recently, several proteins mutated in NPH were found to be implicated in a highly conserved pathway, the so-called DNA damage response (DDR) that senses and signals the presence of DNA damage due to replication stress and can arrest the cell cycle to promote DNA repair. An abnormal DDR could lead to increased apoptosis and epithelial-to-mesenchymal transition of duct cells at the kidney corticomedullary junction and a pro-fibrotic response of surrounding fibroblasts, explaining at least in part the massive fibrosis that usually is more evident than cysts in patients with NPH [59,60].

Brain

Primary cilia were first identified on neuronal cells during electron microscope examinations of brain tissue sections [61,62], but only decades later could the relevance of this observation be fully appreciated. The functional characterization of ciliary genes in cellular and animal models and the dissection of the interplay between the primary cilium and pathways essential for brain development have greatly expanded our knowledge of the role of this organelle in regulating neuronal cell fate, migration, differentiation, and signalling.

In mammals, primary cilia are essential mediators of the Shh pathway of which many components are variably localized within the cilia at different steps of pathway activation [63]. Dysregulation of Shh signalling due to mutations in genes encoding proteins of the pathway results in neural tube closure defects, hydrocephalus, and other midline defects such as occipital encephalocele, corpus callosum defects, and holoprosencephaly [64] which are also part of the ciliopathy spectrum. Moreover, cilia-mediated Shh signalling represents the main proliferative driver for cerebellar granule neuron precursors [65,66], and mutations or conditional removal of distinct genes implicated in this pathway results in cerebellar dysgenesis and hypoplasia, a condition that is often observed in ciliopathies [67–73].

Another key developmental pathway implicated in cerebellar development is the Wnt canonical pathway, which was found to be enhanced by Jouberin, a ciliary protein encoded by the *AHI1* gene. Jouberin knock-out mice display defective cerebellar vermian midline fusion [74,75], and similarly, *AHI1* mutations in human patients lead to a notable constellation of mid-hindbrain malformations with cerebellar vermis hypodysplasia, the so-called 'molar tooth sign' (MTS) [76]. On the other hand, other evidence suggests a negative ciliary regulation of the Wnt pathway [77], or even no Wnt signalling defects [78], indicating that the interplay between primary cilia and Wnt may be more complex than currently appreciated such that the influence on Wnt could vary according to distinct settings. Besides these, other signalling pathways linked to ciliary function include PDGFRα, involved in promoting directional cell migration [79], and Notch, which was found to enhance the ciliary-mediated activation of the Shh pathway [80].

Neuronal cilia of cortical progenitor cells have also been directly implicated in the modulation of proliferation, directional migration, and differentiation of both excitatory and inhibitory neurons in the developing cerebral cortex [81]. This is thought to be mediated partly by Shh signalling itself and partly by receptors of guidance cues such as PDGFR and GPCR, localized on the ciliary membrane of interneurons [82,83]. A key ciliary component implicated in control of neuronal migration is the cilia membrane-associated small GTPase ARL13B, whose ablation results in impaired ciliary localization of specific guidance cue receptors and defective placement of postmitotic interneurons that is also associated with mislocalized ciliary signalling machinery [84,85]. Interestingly, mutations of *ARL13B* typically cause JBTS, a ciliopathy characterized by the MTS and neurological features [86]. ARL13B is also implicated in regulation of membrane biogenesis and cilia length control, a function disrupted in the context of JBTS causal mutations [87]. Overt malformations of cortical development such as polymicrogyria have been reported in a minority of JBTS patients [88,89], but it is possible that more subtle defects of cortical development due to

ciliary dysfunction may contribute to the cognitive defects that are nearly invariably seen in JBTS patients. The lack of decussation of the superior cerebellar peduncles and pyramidal tracts reported in neuropathological as well as diffusion tensor imaging tractography studies of patients with JBTS [90,91] has suggested that defective primary cilia could also impair the process of axonal guidance. However, it is also possible that these crossing defects may be secondary to altered cell fate or survival, and a specific role for cilia in axon guidance still remains to be demonstrated to date [92].

Primary cilia are also thought to play a role in the formation of adult neural stem cells, a pool of neural progenitors within the hippocampal dentate gyrus able to generate neurons during postnatal life [93]. Embryonic ablation of either ciliary genes or components of the Shh pathway, such as Smo, resulted in failed development of radial astrocytes in the dentate gyrus, with subsequent failure of postnatal neurogenesis [94].

Finally, primary cilia have been reported in both the orexigenic and the anorexigenic neurons in the arcuate nucleus of the hypothalamus, implicated in the metabolic regulation of food intake and responses to the adipocyte hormone leptin and the pancreatic hormone insulin. Indeed, the systematic ablation of some ciliary genes from adult mice resulted in hyperphagia and obesity, with increased levels of insulin, leptin, and glucose [95]. On the other hand, the obesity phenotype observed in some ciliopathies could also relate to defective leptin signalling or leptin resistance, and to abnormal modulation of Shh and Wnt signalling, both of which play a role in the regulation of adipogenesis [96].

Retina

In the vertebrate neural retina, cone and rod photoreceptors rely on the outer segment, a highly specialized ciliary organelle capable of detecting light through a complex structure of regularly stacked, photopigment-filled membranous disks oriented along the axis of the incoming light, which are either fully internalized or in continuity with the plasma membrane. The outer segment is connected to the cell body (also termed inner segment) through a thin connecting 9 + 0 cilium anchored to a triplet microtubule basal body derived from the mother centriole between the outer and inner segment [97]. The photoreceptor-connecting cilium corresponds to the ciliary transition zone of primary cilia, and is essential for regulating the flux of specific proteins in and out of the outer segment. This involves mainly disk proteins such as rhodopsin, which are continuously trafficked along the connecting cilium, as well as other proteins that shuttle between the two compartments following changes in ambient lighting [98]. As in other non-specialized primary cilia, anterograde and retrograde protein trafficking is mediated by IFT complexes which are associated with motor proteins that move up and down the ciliary axoneme. Indeed, selective knockdown of various IFT proteins in mice photoreceptors results in the accumulation of ectopic rhodopsin, impaired formation of the outer segment, and increased cellular death [99–101].

Given the complexity of the retinal modified cilium, it is not surprising that mutations of multiple photoreceptor proteins can impact at different levels on its development, maintenance, and functioning, resulting in the phenotype of retinal dystrophy that is a common feature in ciliopathies. Photoreceptor proteins associated with ciliopathies include ALMS1, mutated in LCA and Alström syndrome (a renal ciliopathy often presenting with retinopathy), which has been implicated in the transport of rhodopsin and other proteins along the photoreceptor axoneme [102]; CC2D2A, mutated in retinitis pigmentosa, JBTS, and MKS, which regulates the extension of the connecting cilium and the outer segment [103]; and TMEM67, also causative of a spectrum of ciliopathies with multi-organ involvement, which is involved in membrane disk assembly [104]. However, it is interesting to note that isolated forms of retinal dystrophy or LCA are not invariably ciliopathies and only a subset of the many causative genes of this phenotype are implicated in ciliary assembly or function [105]. In parallel, cilia genes can be associated with both isolated or syndromic (ciliopathy) forms of retinal dystrophy, for example *C21ORF2* and *IFT140* [8,9].

Liver

Primary cilia protrude from cholangiocytes, the epithelial cells lining the biliary ducts of the liver. These form early during development starting from a transient structure, the ductal plate, which appears around the sixth to seventh week of gestation in the region between the branches of the portal vein. Here, hepatoblasts start to differentiate into primitive cholangiocytes and form bile ducts, which are separated by surrounding liver parenchyma by intense mesenchymal proliferation paralleled by enhanced apoptotic processes [106]. Similarly to renal cilia, primary cilia on cholangiocytes function as mechano-, chemo-, and osmo-receptors, which sense biliary lumen flow, composition, and osmolality, and transduce these signals through modulation of intracellular calcium and cAMP [107]. The impaired functioning of primary cilia due to mutations in a ciliary protein results in aberrant remodelling of the ductal plate (so-called 'ductal plate malformation of the liver'), with the formation of abnormal bile ducts that are surrounded by excessive extracellular matrix and often present cystic dilatation [108]. This congenital hepatic fibrosis can remain paucisymptomatic or manifest with severe complications, mainly portal hypertension, cholangitis or cholestasis [109]. Many primary ciliopathies display liver fibrosis including PKD, NPHP, BBS, JBTS, and MKS [45,110].

Pancreas

Primary cilia are also involved in the development and functioning of the pancreas, an organ with exocrine and endocrine functions that comprises distinct cell types,

of which about 15% are ciliated. In particular, primary cilia have been detected on ductal cells as well as α-, β-, and δ-cells in the islets of Langerhans [111,112]. Pancreas development is a complex process that involves several key pathways (Shh, Wnt, TGF-β, Notch, and FGF), all of which are modulated by the functioning of primary cilia. Moreover, primary cilia in adult pancreatic ductal cells have been proposed to sense and transduce signals related to luminal flow, similarly to their counterpart in the kidneys and liver [113]. Correlating with this, ciliary impairment has been associated with pancreatic defects that, as in the liver, are mainly characterized by fibrosis, dysplasia, and the formation of ductal cysts. However, pancreatic involvement in ciliopathies is less frequently documented, possibly because the exocrine and endocrine functions are often preserved despite the underlying structural damage. An exception is in Alström syndrome, in which dysfunction of the β-cells and diabetes mellitus are consistent and typical features [114].

Skeletal system

Cilia-related skeletal phenotypes mainly arise from IFT defects that cause deficiency of the Hedgehog pathways, affecting the growth of the cartilage and bones [115]. Indian hedgehog (Ihh) is a key signalling molecule in the endochondral bone formation responsible for most skeletal components including the ribs and long bones, regulating chondrocyte maturation during this ossification process; mice with disrupted Ihh signalling have shortened long bones and a short and narrow thorax [116,117]. Defective Shh in *Ift88* mouse mutants is thought to underlie their polydactyly and aberrant skull formation through incorrect expression of the downstream GLI effectors that specify digit patterning [118,119].

The IFT system has been well characterized by numerous methods including protein crystallography [5,6]. Ciliopathy associated mutations are found in selected subunits of the IFT retrograde dynein motor and components of the IFT complexes A and B [120,121], or proteins of the basal body and centrosomes likely connected to IFT but with less defined functions [9,120,122,123]. Components of IFT implicated in these diseases transport the transmembrane smoothened (SMO) receptor, a key signal transducer in Hedgehog signalling, along the cilia. In their absence, SMO accumulation to cilia is not sufficient to activate the pathway [124,125]. Disturbed ciliary targeting of SMO is at least partially responsible for the premature differentiation and reduced proliferation of chondrocytes in long bone growth plates that underlie SRPS phenotypes [126].

Links between motile and non-motile cilia functions

The links between motile and non-motile ciliopathies are unclear but increasing knowledge about sensory receptors in motile cilia and the influence of mechanosensory signals on motile cilia in the embryonic node and elsewhere has led to the question of whether sensory functions (chemo- and mechano-sensitivity) can be attributed to motile as well as primary cilia. Clinical ciliopathy studies have reported overlapping features of motile and primary ciliopathy disorders in relation to laterality defects, infertility, and hydrocephalus.

With the unique features of nodal cilia that can move despite sharing the typical 9 + 0 arrangement of non-motile cilia, and the mix of motile and non-motile cilia at the left–right organiser, it is perhaps not surprising that laterality defects, in the form of partial or complete *situs inversus*, are part of the phenotypic spectrum of both motile and non-motile ciliopathies such as JBTS, NPH, and skeletal ciliopathies [127–131]. Interestingly, homozygous mutations in the *NPHP2* gene, which usually cause infantile NPH with *situs inversus*, were found in a fetus displaying these features as well as signs of motile cilia dyskinesia, expanding the phenotypic spectrum of this gene to include motile and non-motile ciliopathies [132].

Lung and airway defects have been reported in BBS, MKS, NPH, and retinal dystrophy patients, but whether these are organ development rather than cilia motility-related problems and whether there is any common aetiology has yet to be proven [133]. Indeed, respiratory motile cilia dysfunction has been excluded in BBS [134]. Syndromic forms of motile cilia disease have been associated with mutations in *RPGR* and *OFD1* (Simpson–Golabi–Behmel syndrome, type 2) but the underlying basis for impaired cilia motility is less clear in these rarer cases where the syndromic features reflect more common phenotypes [135,136].

Phenotypic spectrum of non-motile ciliopathies: more a continuum rather than distinct syndromes

Since the first descriptions of human ciliopathies nearly two decades ago [137,138], the number of disorders falling under the umbrella of primary ciliopathies has significantly increased. Currently, this term includes several syndromes that are clinically diagnosed based on the major organ(s) involved, spanning a spectrum of severity from relatively mild to lethal (Figure 3).

The first group of disorders identified as primary ciliopathies were the cystic kidney disorders, including the two main groups of PKD and NPH. Both ADPKD and ARPKD are characterized by enlarged multicystic kidneys, but they differ by the age at onset (in adult and prenatal life, respectively) and the extent of multi-organ involvement. In fact, ADPKD features multiple cysts in the liver, pancreas, seminal vesicles, and arachnoid membrane, frequently associated with cardiovascular defects (e.g. arterial dilatations/aneurysms and cardiac valve abnormalities), while ARPKD typically presents congenital liver fibrosis [55,139]. While PKDs are extremely rare conditions, juvenile NPH

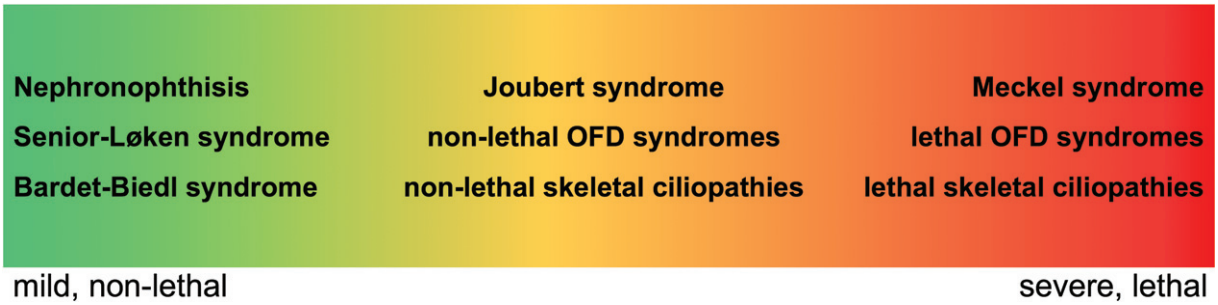

Figure 3. Spectrum of the severity of primary ciliopathies. Distribution of known ciliopathy syndromes across a spectrum of severity.

represents the commonest genetic cause of end-stage renal failure (ESRF) in children. Distinct from PKD, it is characterized by tubular atrophy, irregular tubular membranes, progressive tubulointerstitial fibrosis, and inflammation, leading to the formation of small, hyperechogenic kidneys and occasional cysts restricted to the corticomedullary border, which appears poorly differentiated. Infantile NPHP, with onset of ESRF in early childhood, is much rarer, and combines the tubular atrophy and fibrosis typical of NPH with widespread cysts and kidney enlargement as seen in PKD. SLS is defined by the association of NPH with retinal dystrophy, often in the severe form of LCA [140].

Among the non-lethal ciliopathies, two relevant conditions are BBS and JBTS. BBS is among the mildest ciliopathies, diagnosed by the primary features of cone–rod retinal dystrophy, post-axial polydactyly, obesity (with hypogonadism in males), genital and renal malformations, and intellectual impairment [141]. JBTS is uniquely characterized by the MTS, a pathognomonic constellation of mid-hindbrain defects clearly appreciable on brain imaging. The MTS derives from the association of cerebellar vermis hypodysplasia, thickened and horizontalized superior cerebellar peduncles, and deepened interpeduncular fossa, giving the appearance of a 'tooth' on MRI axial sections at the pontomesencephalic level. This typical pattern can variably associate with defects in other organs, including the kidneys, retina, liver, and skeleton, giving rise to an extremely large spectrum of phenotypes, from relatively mild to severe [142]. At the end of this spectrum is MKS, a lethal ciliopathy characterized by enlarged cystic kidneys, polydactyly, occipital encephalocele, and frequently congenital liver fibrosis [143].

Two other groups of ciliopathies displaying a wide range of severity are skeletal ciliopathies and OFD syndromes. Skeletal ciliopathies comprise at least 16 different subtypes including the lethal SRPS type I–V and syndromes more compatible with life, Jeune syndrome or asphyxiating thoracic dystrophy (ATD), Mainzer–Saldino syndrome (MZSDS), and Ellis–van Creveld syndrome (EVC or chondroectodermal dysplasia). These recessive disorders of skeletal bone growth manifest with short ribs giving rise to a constricted thorax, shortened long bones, and a characteristic trident aspect to the acetabular roof, with or without polydactyly. Cleft lip/palate and defects of the eye, heart, kidneys, liver, pancreas, intestines, and genitalia can also be variably present. Cranioectodermal dysplasia (CED; Sensenbrenner syndrome) is an overlapping ciliopathy with similar genetic origins and skeletal abnormalities that feature craniosynostosis, narrow rib cage, short limbs, and brachydactyly [144]. EVC and CED in addition manifest with variable ectodermal defects affecting the teeth, hair, nails, and skin. Finally, OFD syndromes are a heterogeneous group of ciliopathies (more than 15 forms have been described to date) that share the association of oral, facial, and digital defects, and are clinically differentiated by the occurrence of additional involvement of other organs such as the brain and kidneys [145].

While this classification of ciliopathy diseases into distinct subtypes is widely adopted in clinical practice, it is important to bear in mind that the overlap of clinical features among ciliopathies is striking (see Table 2 in ref 144), often making it difficult to assign a specific diagnosis to a patient. For instance, the MTS may occur in association with short ribs and other skeletal defects, fulfilling the diagnosis of both JBTS and JATD [146–148], or in association with typical features of OFD, defining the so-called OFD VI syndrome, which is classified within both the JBTS and the OFD subgroups [145,149]. Adding further complexity, some patients present anomalies that are typical of multiple ciliopathies: one such example is OFD IV, a condition sharing features of SRPS (shortened long bones, trident appearance of the acetabulum), OFD (lobulated tongue, polydactyly), and MKS (occipital encephalocele, enlarged cystic kidneys, and ductal plate proliferation of the liver) [150].

Genetic basis of non-motile ciliopathies

The clinical heterogeneity of non-motile ciliopathies is mirrored by their genetic heterogeneity, and the recent advent of whole-exome and whole-genome sequencing strategies has impressively accelerated the identification of novel ciliopathy genes even in families underpowered for linkage studies [151]. To date, we know of over 50 genes causative of non-motile ciliopathies, and functional studies have disclosed interesting correlates between the function and the ciliary

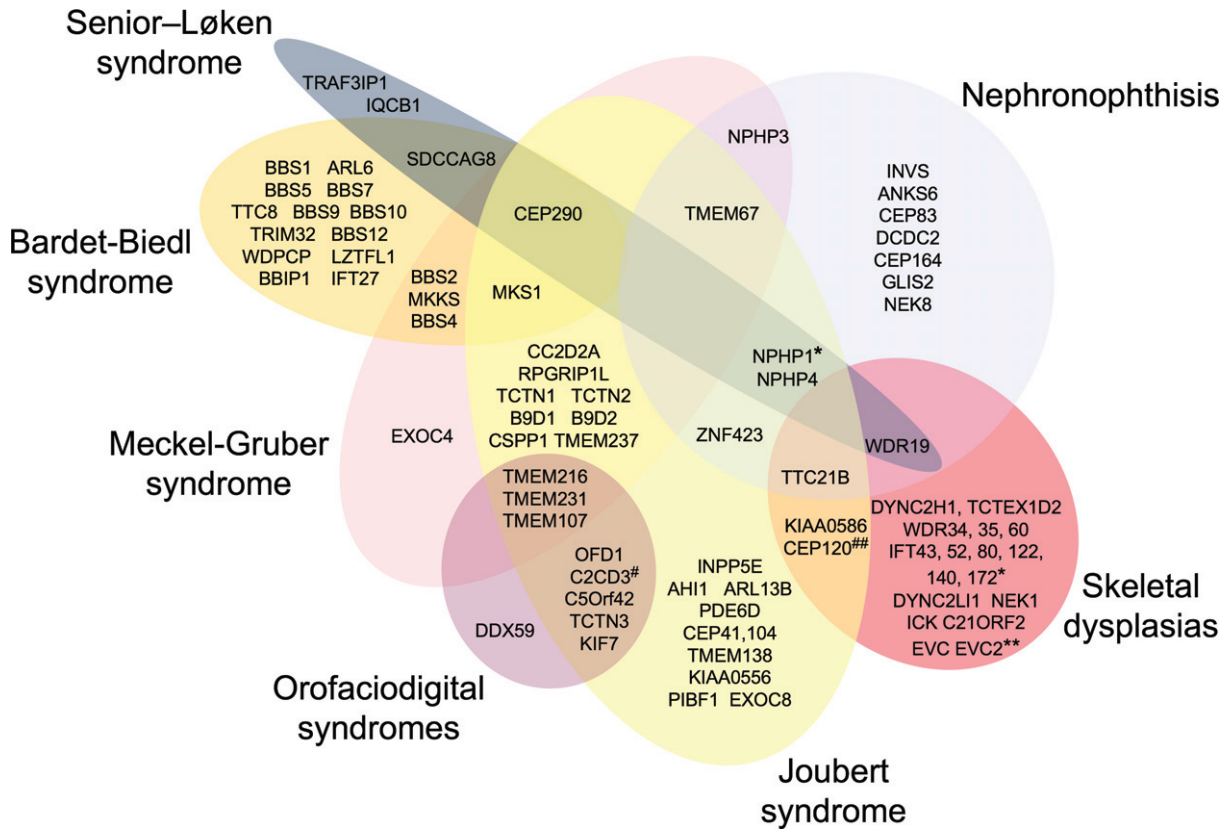

Figure 4. Clinical and genetic variability of primary ciliopathies. Venn diagram summarizing the genetic overlap among distinct ciliopathies. *Also found mutated in a single family with BBS [189,190]. **Also proposed as a novel candidate gene for MKS [180]. #Also found mutated in a single family with skeletal dysplasia [191]. ##Also found mutated in a fetus resembling MKS [192]. Figure extended from ref [193].

domain of the mutated protein and the underlying clinical phenotype. Many proteins were found to cluster in discrete complexes ('modules') bearing specific functions within the cilium. Indeed, most skeletal ciliopathies are caused by mutations in IFT components [152], while the majority of BBS-related proteins form the BBSome that modulates the correct assembly of IFT complexes at the ciliary base and regulates the turnaround from anterograde to retrograde transport at the ciliary tip [153]. Conversely, most proteins mutated in JBTS, MKS, and NPH reside in the transition zone, where they form distinct functional modules (such as JBTS/MKS and NPH complexes), which essentially regulate cilia-related signalling cascades and protein trafficking in and out of the cilium [154] (Figure 1). Protein redundancy and functional interaction between complexes have been demonstrated in distinct *in vivo* models, possibly explaining why mutations in so many genes can result in similar multi-organ pathologies [155,156]. Interestingly, CEP290 was found to regulate the activity of distinct complexes, which could justify the pleiotropic phenotypes associated with its mutations (see below) [157].

Besides gene discovery, next-generation sequencing technologies have also revolutionized genetic diagnosis, allowing us to simultaneously, rapidly, and cost-effectively sequence hundreds of ciliary genes in large cohorts of patients [158]. This has resulted in a more accurate estimate of the mutation frequency, as well as in an unexpected expansion of the phenotypic spectrum of ciliary genes (Figure 4). Interestingly, some genes appear to be very organ-specific; for instance, mutations in *ARL13B* have been identified only in JBTS patients with purely neurological manifestations [86], while to date *IFT80* and *DYNC2H1* are found mutated only in isolated SRP phenotypes [84,85]. Other genes are not so selective but still show a preferential involvement of specific organs and tissues. Some examples are *TMEM67*, nearly invariably associated with congenital liver fibrosis [159–161]; *C5Orf42*, whose mutations cause OFD as well as JBTS with a high prevalence of polydactyly [162,163]; and *IFT40*, mutated in SRPS with a high prevalence of severe kidney diseases [7]. On the other hand, genes such as *CEP290* are extremely pleiotropic, being mutated in a wide spectrum of ciliopathies with defects in the retina, kidneys, liver, and CNS [164]. Similarly, *KIAA0586* mutations are known to cause a relatively mild form of pure JBTS as well as more complex ciliopathy phenotypes, with features of JBTS, OFD, and SRPS [123,147,165–167].

Some genotype–phenotype correlates have been established, as the occurrence of at least one hypomorphic mutation is usually associated with milder phenotypes, while biallelic loss-of-function mutations frequently lead to severe and often lethal disorders [128,160,168,169]. However, such correlates are only in part able to explain this variability, as they are challenged by several pieces of evidence. For instance,

the homozygous deletion of the *NPHP1* gene is a recurrent mutation that is known to cause distinct phenotypes, from isolated NPH to oculo-renal and cerebello-oculo-renal ciliopathies [170–172]. Moreover, significant clinical variability has been reported among siblings carrying the same genetic mutations [173], suggesting the existence of genetic, epigenetic or even environmental modifiers able to modulate their phenotypic manifestation. Some BBS families were found to show 'oligogenic inheritance', as autosomal recessive mutations in a BBS gene had to be associated with a third heterozygous mutation in a distinct gene in order to become penetrant [174,175]. While true oligogenic inheritance has not been confirmed in other ciliopathies, the existence of genetic phenotypic modifiers has been suggested by some sporadic observations, showing a positive correlation between the presence of certain heterozygous variants (e.g. *AHI1* p.R830W or *RPGRIP1L* p.A229T) and the occurrence of neurological, retinal or renal manifestations [76,176,177]. However, these findings required additional confirmation in larger, independent cohorts.

Despite the progresses in gene discovery made in the past decade, a proportion of patients remain without a genetic diagnosis, indicating that a subset of genes still has to be identified. Of note, this proportion varies among different ciliopathies and, most intriguingly, when considering the organs involved. From recent NGS-based screenings and our own personal experience, we consider that BBS appears to be the most solved condition, a genetic diagnosis being reached in about 70–80% of cases [178,179]. Skeletal ciliopathies and MKS have a success rate above 70% [180–182], while this is up to 60% for JBTS [89]. Yet this proportion lowers to about 40% when considering the subgroup of JBTS with kidney involvement, in line with distinct studies reporting a mutation rate only up to 20% in NPH-related ciliopathies [183,184]. From these observations, it seems that genes with major kidney expression have been less characterized than genes involved, for instance, in skeletal or brain development.

Conclusions and future perspectives

The mutation spectrum for motile ciliopathies (PCD, RGMC) has been greatly expanded, allowing an extensive biology-defined stratification of patients into distinct gene and mutation categories. Moreover, new insights into the regulation of multiciliogenesis have arisen and clinically meaningful correlations are emerging to connect the underlying genotype of affected individuals with clinical outcomes across the lifespan of these chronic diseases [48,185,186]. This assists diagnosis that is complicated by clinical heterogeneity, with motile ciliopathies acknowledged to be greatly underdiagnosed [13]. The hope is to move this field more rapidly towards clinical translation, using novel pharmacogenomic approaches to target therapy in a biologically appropriate manner.

For the primary ciliopathies, the impressive clinical and genetic heterogeneity and marked overlap among distinct syndromes present a major challenge for the physicians dealing with these disorders, and it is not unusual that a patient receives different diagnoses from clinicians with expertise in different pathologies. The complex scenario of ciliopathies recalls the ancient anecdote of 'the elephant and the blind men' [187]: six blind men encountered an elephant for the first time and each touched a different part of its body, reaching different conclusions about its nature (a pillar, a rope, a tree branch, a fan, a wall, a pipe) according to the part they had touched (the leg, the tail, the trunk, the ear, the belly, the tusk). Of course, despite each having made an accurate analysis, all had reached false conclusions as they missed the 'big picture'. Similarly, when approaching a patient with a ciliopathy, a common mistake is that of sticking to a specific syndromic diagnosis based on the presence of certain features. This is not a trivial issue, as it bears consequences in terms of management and counselling of patients and their families. While in some cases a syndromic diagnosis is straightforward, clinicians should be aware that, for other patients, the attempt to classify the phenotype within one or other ciliopathy syndromes may be inaccurate, and it would be better to provide a more descriptive diagnosis based on the extent of multi-organ involvement. The increasing availability of large-scale genetic testing including unbiased approaches such as whole-exome or whole-genome sequencing also provides a useful diagnostic tool, as it allows a reclassification of complex ciliopathy phenotypes based primarily upon their genetic defect, and can suggest a potential spectrum of organ involvement according to the gene that is found mutated.

Current 'omic' technologies are also leading ciliary research into novel, intriguing avenues. For instance, an unbiased approach combining affinity proteomics, genetics, and cell biology allowed definition of the 'ciliary landscape', highlighting interactions and protein complexes that could not be revealed otherwise, and which can possibly expand the spectrum of ciliopathies to include other, apparently unrelated disorders [188]. Similarly, unbiased siRNA-based functional genomic screens matched with whole-exome sequencing data led to the identification of novel ciliopathy genes and regulators of ciliogenesis [9,166]. Yet despite the major progress made in recent years, the pathobiological mechanisms underlying the striking variable expressivity of ciliary gene mutations remain largely unknown, greatly hampering the appropriate counselling of families, especially those needing to make reproductive choices. In our opinion, a deeper understanding of these mechanisms represents the greatest challenge ahead in the field of ciliopathy research.

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Author contribution statement

HMM and EMV wrote the manuscript, prepared the figures, edited and revised the manuscript, and approved the final version.

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