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Abstract

Primary cilia are essential signalling organelles found on the apical surface of epithelial cells, where they coordinate chemosensation, mechanosensation and light sensation. Motile cilia play a central role in establishing fluid flow in the respiratory tract, reproductive tract, brain ventricles and ear. Genetic defects affecting the structure or function of cilia can lead to a broad range of developmental and degenerative diseases known as ciliopathies. Splicing is of fundamental importance to the pathogenesis, diagnosis and treatment of ciliopathies. Tissue-specific splicing is particularly important in the highly specialised ciliated cells of the retina, the photoreceptor cells. Ciliopathies can arise both as a result of genetic variants in spliceosomal proteins, or as a result of variants affecting splicing of specific cilia genes. Here we discuss the opportunities and challenges in diagnosing ciliopathies using RNA sequence analysis and the potential for treating ciliopathies in a relatively mutation-neutral way by targeting splicing.

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Suggested reviewers	Emanuele Buratti, Jane Lucas, Franco Pagani, Michael Ladomery, John Holloway, Sebastian Oltean

Submission Files Included in this PDF

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Editor and Reviewer Comments:
-Reviewer 1

- This is a timely and interesting review article that draws attention to the importance of dysregulated pre-mRNA splicing in the pathogenesis of several ciliopathies, providing opportunities for better diagnosis and potentially offering avenues for the development of new therapies. There have been a plethora of review articles on alternative splicing in disease but one focused on ciliopathies is original, and very welcome at this time.

It is a topical, interesting, well researched and well written review; however I feel it could be improved through a series of mostly minor alterations, as indicated in the following list. In general I feel that some topics and points need to be explained in a little more detail.

1. On a general stylistic point, I notice quite a lot of rather short, even one-sentence paragraphs. You might want to consider amalgamating paragraphs or filling out paragraphs that could do with additional detail.

We have addressed this issue by both amalgamating some paragraphs and extending some paragraphs to include additional detail.

2. Lines 29-33 as this is a key point of interest to the reader, can you elaborate by providing more detail about how motile cilia play a role in development.

We have provided more information here (lines 31 – 34)

3. Lines 43-48 this list of ciliopathies could do with being presented in a suitably formatted table.

We have added this information in a new table, now Table 1.

4. 66-71, please clarify, are these all worldwide incidences?

This has been clarified in lines 66 – 76.

5. 74, rather than referring to an OMIM webpage that could change, could you refer to some explicit literature?

We have replaced the OMIM page with a reference, now on line 72.

6. 90-91 can you expand here and explain in more detail the findings of reference [13].

More detail has been added here, now in lines 100 – 111, 113 – 115 and 117 – 122.

7. 93, granted the human retina displays extensive transcript diversity. This connects to the final paragraph of the section 124-127 "..aberrations of splicing may be more likely to have a physiological impact on retinal photoreceptor cells". I don't feel that this claim is sufficiently justified here - yes I appreciate that reference [14] is pointing to a plethora of alternative splicing events, but how does transcript diversity in the retina compare to that in other tissues? so perhaps you could elaborate here to strengthen the point.

We have expanded on this section, now in lines 125 – 141.

8. 98-107 when describing these splice isoforms can you be more precise and explain the exact nature of each splice isoform - the specific mode needs to be stated; eg cassette exon, alternative 3' splice site etc. This list of isoforms could perhaps also benefit from being summarised in a table too.

We have added more detailed information and added additional examples, in lines 147 – 160.

9.156-159, this section on DDX59 is rather short - there seems to be some interesting recent literature on DDX59, so could you perhaps elaborate.

We have provided additional detail here, from lines 204 – 225.

9. 173 please explain exactly how exons 9 and 12 are incorrectly spliced.

We have provided additional detail, lines 306 – 309.

10. 180-213 most of this section seems to be interested on the challenges associated with characterising splicing mutations. This is a fair enough point, but perhaps this section (on splicing in ciliopathies etc) could be re-balanced, with less emphasis on the challenges of splicing mutations in general, and more detail on the ciliopathy associated splicing mutations.

We have added more detail on ciliopathy-associated mutations (lines 243 – 273, 280 – 287, 289 – 304 and 335 – 341) to balance this section.

12.224-227 avoid one-sentence paragraphs.

We have amalgamated several shorter paragraphs into one.

13. 261-307 this section on the technical challenges of interpreting RNASeq data is very interesting but its pertinence to the main topic (splicing in ciliopathies) really does need to be strengthened. Could additional details about the NPHP3 gene and its faulty splicing in relation to ciliopathies be provided. Perhaps additional examples could also be provided, relating the technical challenges of RNASeq data to the splicing of cilia-associated genes.

We have added more ciliopathy-specific detail (lines 361 – 374, 390 – 405 and 407 – 408 and 413 – 434)

14. 312-3019 are these weblinks to a specific clinical trial an appropriate reference? (more on the next page).

We have replaced these with shorter URLs.

15. 338 add some text to explain how Eteplirsen works, to strengthen the point.

We have added additional detail here (lines 515 – 527)

16. 356-359 another one-sentence paragraph. Please provide more details on the USH2A trial.

We have amalgamated several shorter paragraphs into one, and added details on the USH2A trial (lines 548 – 553).

17. 368 please explain the mechanism; how does the ASO rescue the splicing defect in CEP290? (presumably with reference to Figure 5?).

We have added detail here (lines 558 – 564) and referred to figure 4 (renumbered from Figure 5).

18. 402-404 please provide more details on how the approach detailed in reference [76] works.

Additional detail has been added here (lines 600 – 610).

19. 417-440 conclusion, too many one-sentence paragraphs in this final section.

We have amalgamated several shorter paragraphs into one.

20. A general point: are all of the figures referred to in the text?

Yes.

21. Figure 3 I find this figure a little underwhelming; could it be augmented, even with some simple labeling, making its relevance to ciliopathies clearer to the reader.

We have removed this figure as we agree that it added little to the paper.

22. Figure 4, I am not sure that showing different ASO chemistries is really necessary in this review. A better figure would be one that summarises how different ASOs have been used to tackle splicing defects in ciliopathies.

This figure (now figure 3) has been revised

Reviewer 2

Dr. Baralle and colleagues present a very interesting overview of current knowledge on the impact of splicing on ciliopathies. The review starts with a description of cilia and how non-functional cilia cause inherited diseases called ciliopathies. This is followed by a brief discussion of alternative splicing during human development and a description of alternative splicing isoforms detected in the retina. Next, authors discuss how mutations in genes that encode components of the splicing machinery are associated with pathology of the retina. Then, authors address the impact of genetic variants that may alter splicing regulation and the identification by RNA seq of these variants in genes associated with ciliopathies. Finally, recent advances in gene therapy for retinal diseases are described, and the potential of targeting splicing for future therapies is discussed.

The following issues should be addressed in a revised version of the manuscript.

1. In the abstract, the sentence "splicing is of fundamental importance to the pathogenesis, diagnosis and treatment of ciliopathies" is clearly an overstatement that needs correction. As discussed in more detail below, the experimental data available raises some possibilities, but the actual mechanism underlying the importance of splicing is not yet demonstrated for particular ciliopathies. Also the sentence "tissue-specific splicing is particularly important in the highly specialized ciliated cells of the retina" is not correct. Tissue-specific splicing is highly important in any specialized cell of the human body.

   We have made these corrections in the abstract and main text.

2. Section "The importance of alternative splicing in ciliated cells". This section should start with a more elaborate description of the splicing mechanism, and what is the physiological relevance of tissue-specific alternative splicing. Several recent reviews have covered this topic and should be cited. The sentence "splicing is particularly important for regulation of the splicing process itself," must be explained giving mechanistic detail and appropriate references. Also the sentence "and for regulation of connecting cilium and outer segment biogenesis, which occur from 12-18 weeks post conception" needs to be better explained, mentioning specific experimental data reported in reference 13. It is important to know that the human retina displays multiple splicing isoforms, but what is their physiological relevance? What experimental models can be used for functional studies? These are current knowledge gaps that could be discussed. Again, the sentence "Thus, aberrations of splicing may be more likely to have a physiological impact on retinal photoreceptor cells than other cell types of the body" is flawed, there is no functional data to support such statement.

   We have added a description of the mechanism of splicing and the physiological relevance of tissue-specific splicing (lines 79 – 87 and 89 - 96)

   We have added detail to lines 100 – 122 to explain "splicing is particularly important for regulation of the splicing process itself," and "and for regulation of connecting cilium and outer segment biogenesis, which occur from 12-18 weeks post conception"

   We have discussed retinal isoforms, their physiological relevance and studying them in more detail in lines 125 – 141.

   The sentence "Thus, aberrations of splicing may be more likely to have a physiological impact on retinal photoreceptor cells than other cell types of the body" has been removed.

3. Section “retinal ciliopathies associated with spliceosomal proteins”. The findings reported in ref 27 raise the possibility that the mutant proteins play a role in ciliogenesis independent of their function in splicing. This argues against the general statement in the Abstract that “splicing is of fundamental importance to the pathogenesis… of ciliopathies”.

   The statement “splicing is of fundamental importance to the pathogenesis… of ciliopathies” has been edited in the abstract.

4. Section “splice variants in ciliopathy genes”. It would be useful for the non-specialist reader to start this section with an overview of splicing regulation and explaining how splice mutations may interfere with normal splicing regulation, highlighting that this process is distinct from alternative splicing. It is also important to mention that splice variants occur in any gene, therefore there is nothing special about splicing in ciliopathies.

   We have added a general overview of splicing regulation and splicing mutations (lines 227 – 239)

5. Section “Treating ciliopathies by targeting splicing”. A reference to NusinersenTM (approved for treatment of Spinal muscular atrophy) should be included.

   We have added information regarding nusinersen at lines 514 – 527.

Splicing in the pathogenesis, diagnosis and treatment of ciliopathies

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Abstract

Primary cilia are essential signalling organelles found on the apical surface of epithelial cells, where they coordinate chemosensation, mechanosensation and light sensation. Motile cilia play a central role in establishing fluid flow in the respiratory tract, reproductive tract, brain ventricles and ear. Genetic defects affecting the structure or function of cilia can lead to a broad range of developmental and degenerative diseases known as ciliopathies.

Splicing contributes to the pathogenesis, diagnosis and treatment of ciliopathies. Tissue-specific alternative splicing contributes to the tissue-specific manifestation of ciliopathy phenotypes, for example the retinal-specific effects of some genetic defects, due to specific transcript expression in the highly specialised ciliated cells of the retina, the photoreceptor cells. Ciliopathies can arise both as a result of genetic variants in spliceosomal proteins, or as a result of variants affecting splicing of specific cilia genes. Here we discuss the opportunities and challenges in diagnosing ciliopathies using RNA sequence analysis and the potential for treating ciliopathies in a relatively mutation-neutral way by targeting splicing.

The cilium in inherited human disease

The primary cilium (**Figure 1a,b**) is the cell's central signalling organelle and is fundamentally important for normal development and physiology [1]. Cilia are found throughout the body on the apical surface of most epithelial cells, where they play roles in mechanosensation and chemosensation. A highly specialised primary cilium, the photoreceptor cilium (**Figure 1c,d**), is found on the photoreceptor cell of the retina. This photoreceptor cilium contains proteins which coordinate light sensation [2]. Motile cilia (**Figure 1e,f**), which have a different ultrastructure from primary cilia allowing them to beat and establish fluid flow, also play an essential role in development, in the establishment of left-right body asymmetry. At the embryonic node in early development, a mixed population of motile and non-motile cilia are found. The motile cilia establish leftward flow to move left-specific morphogens to the left-hand side of the developing embryo, defining this as the left. In developed mammals, motile ciilia play a crucial role in movement of mucus in the respiratory tract, ova along the Fallopian tubes, cerebrospinal fluid in the ventricles of the brain, and fluid in the middle ear [3].

When cilia do not form or function properly, a range of inherited conditions called ‘ciliopathies’ arise [4]. These are separated into the non-motile and motile ciliopathies. Due to the widespread importance of the primary cilium across multiple tissues, the non-motile ciliopathies tend to be syndromic conditions, affecting multiple organs. Common features of syndromic non-motile ciliopathies are neurodevelopmental defects (**Figure 2a-c**), skeletal defects (**Figure 2d,e**), obesity (**Figure 2f**), cystic kidney disease (**Figure 2g,h**), and inner ear problems leading to sensorineural hearing loss. Polydactyly (**Figure 2i,j**) and retinal dystrophy (**Figure 2k,l**) are particularly common across syndromic ciliopathies. The non-motile ciliopathies can be broadly classified into the

neurodevelopmental ciliopathies, such as Joubert (JBTS) and Meckel-Gruber syndrome (MKS); the kidney ciliopathies such as polycystic kidney disease (PKD) and nephronophthisis (NPHP); the skeletal ciliopathies such as short-rib thoracic dystrophies (SRTD) and orofaciodigital syndrome (OFD); the obesity ciliopathies such as Bardet-Biedl syndrome (BBS) and Alstrom syndrome (ALMS) and the isolated retinal ciliopathies, including subtypes of retinitis pigmentosa (RP), Leber congenital amaurosis (LCA) and cone-rod dystrophy (CORD) (**Table 1**). Defects in motile cilia lead to a group of disorders termed primary ciliary dyskinesia (PCD) (**Table 1**). PCD is a clinically heterogeneous group of disorders which may include recurrent respiratory infections, progressive upper respiratory problems and loss of lung function, subfertility, infrequent hydrocephalus, situs defects (which may be linked to heart disease) and hearing problems (**Figure 2m-p**) [5].

The ciliopathies are widely genetically heterogeneous (**Table 2**). The vast majority of these conditions are inherited in an autosomal recessive manner, but there are also examples of X-linked inheritance such as X-linked JBTS and X-linked oro-facio-digital syndrome (OFD) associated with *OFD1* mutations. However, the single most common ciliopathy is autosomal dominant PKD (AD-PKD), 85% of which is caused by mutations in *PKD1* [6] (**Table 3**). AD-PKD is the most common cause of end-stage renal failure, and affects between 1:1000 and 1:4000 individuals in the EU, making it one of the most common genetic diseases in humans, and the most common cause of end–stage renal failure [7]. Non-syndromic retinal ciliopathies are also relatively common. Retinal dystrophies collectively affect around 1:3000 people worldwide [8]. The most common of these is RP, which affects around 1:3500 people worldwide [9]. LCA affects around 1:50,000 people and CORD affects around 1:40,000 people worldwide. Around one third of the genetic causes of retinal dystrophy encode proteins of the photoreceptor cilium [10]; **Table 2**), so non-syndromic retinal ciliopathies can be estimated to affect 1:6000 individuals in the general population worldwide (**Table 3**). The recessive ciliopathies are individually much rarer but collectively common, with an incidence rate from 1 in 15-30,000 (USH) to 1 in 100,000 (JBTS, BBS, MKS and ALMS) worldwide (**Table 3**). PCD affects around 1 in 10,000 people worldwide (**Table 3**) and is genetically heterogeneous, with 38 known genetic causes [11] (**Table 2**). As with non-motile ciliopathies, the majority of genetic subtypes of PCD are inherited in an autosomal recessive manner, but X-linked forms caused by mutations in the *RPGR* or *PIH1D3* genes have been reported. Collectively, ciliopathies can be estimated to affect between roughly 1:700 and 1:2000 people in the general population worldwide [12] (**Table 3**).

The importance of alternative splicing in ciliated cells

The vast majority of human genes contain more than one exon, divided by introns, and splicing is the process by which introns are removed from a pre-mRNA and exons joined to make a mature mRNA. This process is catalysed by a large protein and RNA complex known as the spliceosome [13]. The spliceosome is composed of 5 small nuclear RNAs (snRNAs), U1-U5, and many proteins, together making 5 snRNPs. In the process of splicing, U1snRNP recognises and binds the splice donor site (the 5' splice site), and promotes the binding of U2snRNP to the branch site. Independently of this, the U4/U6.U5 tri-snRNP forms in the cell, and is recruited to the pre-mRNA, where U6snRNP replaces U1snRNP. This forms the catalytically active spliceosome, which excises the intron and joins the exons through two transesterification reactions [13]. Alternative splicing is a process central to the functioning of all human cells, with around 95% of multiexon genes undergoing alternative splicing [14, 15]. Alternative splicing involves differential use of 5' (splice donor) and 3' (splice acceptor) splice sites, differential inclusion or exclusion of exons (including mutually exclusive exons) and differential

intron retention/exclusion [15]. Alternative splicing controls fundamental cell processes including protein transport across membranes [16, 17]. Differential transcript expression and alternative splicing is highly tissue-specific. Indeed, patterns are more similar between the same tissues of different species than they are between different tissues of the same organism [18]. Such differential splicing drives different developmental processes across different organs and tissues [19] and contributes to the specific functions of mature cells such as neurons [20, 21]. Ciliated cells are no different from other cells in this regard. However, one particular ciliated tissue deserves special consideration here, for the complexity of its splicing profile; the retina.

Gene expression is extremely heterogeneous early in eye development, which begins at 3.5 weeks post conception (PCW) and continues until 5 months after birth [22]. According to gene expression patterns, there are 3 broad phases of eye development; developing eyes from 4.6-7.2 PCW; developing retinae from 7.7-10 PCW; and developing retinae from 12-18 PCW [22]. Peak expression of genes involved in connecting cilium and photoreceptor outer segment biogenesis occur from 12-18 PCW [22]. The photoreceptor cells in particular display a 'switch-like' splicing pattern, which involves a high rate of inclusion of exons which are not included in transcripts in any other cell type of the body [23]. Whilst photoreceptor cells are sensory neurons, their splicing pattern is not controlled by typical sensory neuron splicing factors, but rather by Musashi 1 (MSI1) protein [23]. Before photoreceptor outer segment development, there is a high rate of specific splicing of primary cilia genes, suggesting that the splicing of these directs the development of the outer segment from a regular primary cilium [23]. Patterns of splicing are highly dynamic through eye development, with variable rates of retained introns, skipped exons, alternative 3′ splice sites, alternative 5′ splice sites and mutually exclusive exons seen in transcripts at different stages of eye development. At 12-18 PCW, significantly different splicing of ciliary genes and genes involved in the RNA splicing process itself is seen in retina, compared to 7.7-10 PCW [22]. This suggests that alternative splicing is particularly important for regulation of the splicing process itself from 7.7-10 PCW, and for regulation of connecting cilium and outer segment biogenesis, from 12-18 PCW. The observation that genes involved in pre-mRNA splicing are significantly differentially spliced at 7.7-10 PCW was confirmed in analysis of similar expression data deposited by an independent group [24]. The authors of this paper suggest that this window of retinal development (7.7-10 PCW) is the stage at which a new specific splicing programme is being established for the next developmental stage, and this explains the enrichment of differentially spliced pre-mRNA splicing genes at this stage of retinal development [22]. The adult human retina also displays extensive transcript diversity. Deep RNA sequencing (300 million reads per samples) of three human retinae identified 79,915 novel alternative splicing events, including 29,887 novel exons, 21,757 3' and 5' alternative splice sites, and 28,271 exon skipping events, and 116 potential novel genes [25]. A large expression study of 50 human retinae confirms the finding that around 50% of transcripts in the retina differ from the GENCODE reference transcriptome, including from 206 putative novel genes [26]. Transcript expression varies across anatomical locations in the retina, both in different cell types [27, 28], and different regions (nasal/temporal and central retina/peripheral) [29]. In particular, there is greater transcript diversity in the neural retina, with around 15,000 alternative splice events are seen in the neural retina, compared to around 10,000 in the retinal pigment epithelium (RPE), choroid and sclera [28]. This is in keeping with the general observation that neural tissues (and testes) exhibit higher transcriptome complexity than other tissues [30-32]. However, it is difficult to make a direct comparison of splicing complexity in the retina compared to other tissues, because the retina has historically been neglected from large expression studies such as GTEx [33] due to difficulty in

obtaining high quality human retinal samples. The physiological relevance of novel retinal genes and isoforms remains to be fully understood, but analysis of novel genes co-expressed with rhodopsin (*RHO*), followed by gene ontology enrichment analysis suggests roles in visual perception, photoreceptor outer segment membrane function, cilium structure and function [26]. Advances in human stem-cell derived retinal organoid culture (discussed later) provide a valuable experimental model for further studies into the function of specific retinal transcripts.

Many genes linked to human retinal disease are known to have retinal-specific isoforms. These genes are alternatively spliced in developing and mature retina, showing different patterns of splicing at different points of development [22]. *ARL6* (BBS3), which can be associated with BBS and non-syndromic retinal dystrophy, has a retina-specific splice variant BBS3L which includes an extra 13bp coding exon near the 3' end (cassette exon). This shifts the reading frame, removes 7 amino acids from the C-terminus of the protein and adds 15 novel amino acids in their place [34]. *MAK* encodes a transcript which includes exon 12 (cassette exon) which is only expressed in the retina and *CEP78* has three retinal isoforms, which include cassette exons 15 and 17 [35]. *RPGRIP1* has a novel retinal transcript which uses an alternative 5' splice site for exon 13 and skips 33 nucleotides from the 5' end of canonical exon 13. This isoform including exon 13d is also expressed at a low level in liver, pancreas and placenta [36]. *TTC8*, which is mutated in BBS type 8, has a retinal-specific isoform which includes a cassette exon, named exon 2a, which was not identified until studies were undertaken in patients with RP, who were found to have -2 splice acceptor site mutation in intron 1 of *BBS8* causing their disease [37]. Studies have confirmed that this splicing mutation, which specifically causes exon skipping of the retinal-specific cassette exon 2a, causes RP due to tissue-specific expression of this transcript exclusively in photoreceptor cells [38]. *BBS5*, which is mutated in BBS, has a retinal specific isoform in most vertebrates. In the mouse, this is formed from the use of cryptic splice sites in intron 7, producing a transcript which produces a truncated protein with a novel C-terminal end [39]. Of particular relevance to human disease is *RPGR*. Mutations in *RPGR* account for 70-90% of XL-RP cases, and 10-20% of all RP cases [40]. There are at least twelve different isoforms of *RPGR*, including two major retinal protein isoforms of RPGR; RPGR(1-19) and RPGR(1-ORF15) [41]. RPGR(1-ORF15) is a retina-specific protein isoform which includes a large 3' terminal exon (ORF15). The retinal-specific terminal exon ORF15 is mutated in 60% of XL-RP patients [42]. Most mutations are small deletions or nonsense mutations leading to premature termination of translation and truncation of the protein, but some affect splicing [42, 43]. Outside of ORF15, splicing mutations are more common than changes in the coding sequence [44, 45]. A +1G>T splice site mutation in intron 5 of *RPGR* has been reported in a family with RP and recurrent respiratory infections [46]. G>T transversion at nucleotide 1164 of intron 15 which may create donor splice site has been reported in one family with atrophic macular degeneration [47]. At the time of writing, 11 splice site variants in *RPGR* are deposited in the clinical variant database ClinVar [48], but only 1 is annotated as 'pathogenic'. This suggests that there is current difficulty in assigning pathogenic status to variants potentially affecting splicing, and splice variants in *RPGR* may be a more common cause of disease than is currently appreciated.

Splicing in the pathogenesis of ciliopathies - retinal ciliopathies associated with spliceosomal proteins

Mutations in genes encoding proteins involved in pre-mRNA splicing, including *PRPF3*, *PRPF4*, *PRPF6*, *PRPF8*, *PRPF31*, *SNRNP200*, *DHX38*, *CWC27* and *RP9*, are the second most common cause of autosomal dominant RP and a minor cause of autosomal recessive RP. 17 years since the identification of *PRPF8*

as a cause of RP, it remains unclear why mutations in pre-mRNA splicing factors cause a phenotype restricted to the retina. A whole genome siRNA knockdown screen for ciliogenesis regulators in a ciliated kidney cell line identified a novel specific role of pre-mRNA splicing factors *PRPF6*, *PRPF8* and *PRPF31* in ciliogenesis [49]. The same observation was made in an independent reverse genetic screen [50]. Further investigation showed that these proteins localise to the base of the photoreceptor cilium, suggesting that these proteins have a role beyond splicing, and classifying these conditions as retinal ciliopathies [49].

Further study in retinal organoids and RPE derived from iPSCs from patients with *PRPF31* mutations show decreased efficiency of splicing in an E1A minigene reporter assay [51]. RPE from patient iPSCs also show a substantial downregulation of SART1, a U5 snRNP protein important for the formation of the pre-catalytic spliceosomal B complex, but no changes in the expression of the U5 protein PRPF8 or the U4/U6 protein PRPF4. Retinal organoids from patients showed differential expression of actin cytoskeleton, ciliary membrane, primary cilium, photoreceptor inner and outer segment, axon terminal and phototransduction proteins. In terms of differential splicing, retinal organoids from patients with *PRPF31* mutations showed an enrichment of mis-spliced centriole and microtubule organisation genes, with skipped exons, retained introns, alternative 5′ and 3′ splice sites, and mutually exclusive exons. In both RPE and retinal organoids derived from *PRPF31*$^{-/-}$ patients, the most significantly mis-spliced genes were genes involved in pre-mRNA and alternative mRNA splicing via the spliceosome. This suggests that ciliogenesis, cilium function, and pre-mRNA splicing are all regulated by alternative splicing in the retina, and this is defective in patients carrying *PRPF* mutations [51].

Other ciliopathy genes have been linked to RNA metabolism, such as *DDX59*, mutated in OFD [52-54]. DDX59 is a DEAD-box RNA helicase, of which there are more than 40 in humans [55]. DEAD-box RNA helicases can unwind short segments of double stranded RNA [56] and are involved in remodeling RNPs [57] for pre-mRNA splicing, RNA nuclear export, and ribosomal biogenesis [58]. Homozygous deleterious missense variants in the helicase ATP binding domain or helicase C terminus of DDX59 are associated with OFD [52]. These pathogenic variants in *DDX59* in OFD patients are associated with normal DDX59 cellular localisation, in a punctate distribution across the nucleus and cytoplasm, and normal ciliogenesis but abnormal ciliary signaling [52]. In *DDX59*$^{-/-}$ patient fibroblasts, cilia grew normally but did not respond appropriately to Hedgehog pathway stimulation, measured by Gli1 expression levels after treatment with Smoothened agonist (SAG) [52]. Variants causing loss of the stop codon in *DDX59* are also associated with OFD, with additional features [53]. Null variants in *DDX59* are associated with a more severe phenotype of OFD with complex neurological involvement, including structural brain anomalies, seizures and global developmental delay [54]. The structural brain abnormalities are similar to those seen in CADASIL syndrome, caused by mutations in the *NOTCH3* gene, leading to speculation that DDX59, through regulation of the primary cilium, plays a role in notch signalling regulation [54]. Loss-of-function *Drosophila* mutants of the homologue of *DDX59* display severe neurodevelopmental defects including gross disorganisation of the peripheral nervous system during development, loss or incomplete ventral nerve cord and shortened lifespan [54]. The data on DDX59 would seem to suggest that this protein, with roles across multiple processes in RNA metabolism, is crucially important for normal embryonic development, in particular, formation of midline structures. The exact molecular mechanism of the role of DDX59 in ciliary function remains

unclear and requires further study but supports the hypothesis that RNA processing is of core importance to ciliary function.

Splicing in the pathogenesis of ciliopathies – splice variants in ciliopathy genes

Normal pre-mRNA splicing and alternative splicing confer healthy diversity and complexity to a range of cellular functions. Splicing is a tightly regulated process, controlled by a range of protein and RNA splicing regulators [59]. Recognition of splice sites depends on conserved sequences around splice sites, and in introns and exons [60]. At each splice site a consensus sequence at the DNA level defines the intron/exon boundary, and the branch site in the intron defines the location of binding of the 5' splice site to the intronic sequence to form the lariat before intron removal [61, 62]. As well as the consensus splice site sequences and the branch point, sequence-specific exonic splice enhancers (ESEs) and intronic splice enhancers (ISEs)[63] promote splicing, and sequence-specific exonic splice silencers (ESSs)[64] and intronic splice silencers (ISSs) [65] inhibit splicing. In spite of tight regulation of the splicing process, specific inherited genetic variants which disrupt the consensus splice site, branchpoint, ESE, ISE, ESS or ISS can lead to aberrant splicing. Such aberrant splicing may constitute the abnormal use of 3' or 5' splice sites, aberrant inclusion of introns or exon skipping and contributes to abnormal cell functions and a range of human diseases [66, 67].

At least 188 different genes can cause a syndromic or non-syndromic ciliopathy phenotype (**Table 2**). Pathogenic splice variants have been reported in more than half (103/188 i.e. 54.79%) of the known ciliopathy genes (**Table 2**). Pathogenic splice variants are particularly common in certain genes. For example, 25% of all published disease-causing variants in *MKS1* affect splicing, and 44.4% of published disease-causing variants in *RSPH1* affect splicing (**Table 2**) [68, 69]. In the case of these two genes, splicing variants are more commonly associated with disease because they cause a loss-of-function, and such loss-of-function variants are sufficient to cause disease whilst missense variants tend to be tolerated and do not cause disease, or cause a less severe disease. Null variants in *MKS1* are a common cause of MKS [70], whilst null variants in *RSPH1* cause a mild form of PCD [71]. Missense variants in *MKS1* have been reported as a very rare cause of BBS [72], but missense variants in *RSPH1* have not been reported as a cause of disease. Similarly to *RSPH1*, loss-of-function variants (but not missense variants) in *CCDC114* cause PCD, and pathogenic loss-of-function splice variants in this gene are common [73]. There are several other ciliopathy disease genes where splice variants are slightly more common because only loss-of-function variants cause disease and missense variants have not been reported as causing disease. These tend to be associated with the severe ciliopathies, and include; *CEP104* and *TMEM237* which cause JBTS [74, 75]; CSPP1 which causes MKS and JBTS with occasional features of SRPS [76-78]; *CCDC39*, *CCDC40*, *HYDIN* and *DNAI2* which cause PCD [79-81]. Occasionally, pathogenic splice variants cause less severe disease than nonsense or frameshift mutations, because the transcript produced by the splice variant remains in-frame. For example, study of the spectrum of mutations in *OFD1* shows that missense and particular splice site mutation (c.2260 + 2 T > G at splice donor site of intron 16, leading to 513bp in-frame deletion) are associated with OFD in males [82], whereas this X-linked condition is usually associated with male lethality in the case of null mutations, including splicing mutations causing frameshifts [83]. A splice site mutation in *TCTN3* which causes in-frame skipping of exon 7 causes JBTS, whereas nonsense or frameshift mutations cause MKS [84]. Pathogenic splice variants are also common in *C8orf37*, which causes BBS, CORD and RP. Particular splice mutations in this gene, such as c.156-2A>G are associated with polydactyly along with CORD [85-89]. It has been postulated that splicing variants impact the transcriptional profile of disease genes

in a more highly variable manner than other types of genetic variant, and due to tissue-specific transcript expression, this could partly account for the broad phenotypic variability observed in ciliopathies, even within families, such as MKS associated with *MKS1* variants [68]. This could help to explain the very broad phenotypic spectrum of conditions from fetal encephalocele to Joubert-related syndrome to OFD, associated with variants in *C2CD3*, which are frequently splice-altering variants which cause frameshifts in the transcript(s) [90]. Around 20% of cases of Joubert syndrome are caused by variants in *AHI1*, of which around 15% are splice site mutations [91]. *BBS1*, the most common cause of BBS (in around 40% of cases) can be associated with mutations at splice donor site in exon 4 (432+1G>A) [92]. The mechanism underlying the higher frequency of splicing variants in certain genes than others remains unclear. Individual splicing changes are also common in certain genes, such as *CEP290*, with the same variant observed repeatedly in many individuals with disease. A deep intronic *CEP290* variant (c.2991+1655A>G), which creates a strong splice-donor site and inserts a cryptic exon in the *CEP290* messenger RNA, is detected in 21% of all LCA patients [93]. 60 -90% of LCA patients with *CEP290* mutations have at least one c.2991+1655A>G allele [94-96]. Similarly, a deep intronic variant in *USH2A* (c.7595-2144A>G) which introduces a novel splice donor site in intron 40, leading to insertion of a pseduoexon, PE40, [97] is the second most common cause of USH type 2A, with a frequency of 4% [98]. Three other deep intronic variants in *USH2A* have been reported as causes of USH type 2A, but these are less common [99]. Variants in *USH2A* are one of the most common causes of ARRP, but whilst splice-site variants in *USH2A* have been reported as a cause of RP, to date no deep intronic variants in *USH2A* have been reported in autosomal recessive RP (AR-RP) patients [100, 101]. A founder mutation, a 29-bp deletion in intron 15 of *MKS1*, is a common cause of MKS in European (and especially the Finnish) populations (the so-called 'Finn-major mutation') [70]. This particular splice mutation is associated with a form of MKS involving skeletal defects, which are rarer in other forms of MKS [102]. The most common genetic cause of MKS, *TMEM67*, has two splice founder mutations in the Pakistani population, (c.1546 + 1 G > A and c.870-2A > G)[103]. A +3 splice donor site mutation at exon 11 of *BBS1*, leading to use of an alternative cryptic donor site within the exon, is a common cause of BBS in the Faroe Islands due to a founder effect [104]. This variant is associated with severe, early onset retinal dystrophy, earlier than that seen in patients with different *BBS1* mutations [104]. In type 2 BBS, a c.472-2A>G splice acceptor site mutation in *BBS2* is a common disease variant in the Hutterite population [105]. The c.742G>A variant, abolishing the consensus splice donor site of exon 7, in *CCDC114* is a PCD founder mutation in the Dutch Volendam population [73] and an intron 2 c.921+3_6delAAGT splice donor mutation in *RSPH4A* is a common cause of PCD in individuals of Hispanic descent due to a founder effect [106]. A 3bp insertion at the exon 1/intron 1 splice donor site in *DNAI1* is a common variant in the Caucasian population, accounting for up to 55% of *DNAI1*-associated cases of PCD in white Europeans, with up to 82% of PCD patients having at least one of these alleles [107]. As *DNAI1* mutations account for up to 9% of PCD cases, this particular variant is significant in terms of its contribution to disease in this population [107]. There have also been reports of common exonic variants affecting splicing. A common Jewish founder mutation in *MAK*, caused by an Alu insertion in exon 9, leads to exon skipping of exons 9 and 12 from the final processed transcript. Exon 12 is a retina-specific exon which is important for specific protein function in the retina, and loss of this exon in patients carrying this Alu insertion leads to RP [108]. A non-synonymous exonic variant in *NPHP3* has also been shown to cause ciliopathy NPHP through aberrant splicing. The synonymous variant c.2154C>T; p.Phe718=, 18 base pairs from the exon–intron boundary within exon 15 of *NPHP3* causes skipping of exon 15 [109].

Genetic diagnosis of ciliopathies is largely achieved using gene panel or targeted exome sequencing. In less genetically diverse cohorts, diagnostic yields of up to 85% can be achieved [110]. However, typical diagnostic yields of 42.4% to 67.6% are achieved for motile ciliopathy patients [111, 112] and 55 to 62% for non-motile ciliopathies [113, 114]. It is likely that underestimation of the contribution of splice defects to the pathogenesis of ciliopathies contributes to limited diagnostic yields. Early estimates suggested that around 15% of pathogenic variants affect splicing [115]. However, intronic variants affecting splicing are often overlooked due to perceived technical difficulties in proving the pathogenicity of these variants. Indeed, when we reviewed pathogenic splice variants in ciliopathy genes reported in the literature (as collated in HGMD), [116] we found that on average around 17.5% of variants affect splicing. However, when we compared this to variants in clinical variant interpretation database ClinVar [48], only 6% of pathogenic variants affected splicing (**Table 2**). Many more variants affecting splicing were assigned 'uncertain significance' status. It seems clear that there remains uncertainty when assigning pathogenic status to splice variants, and this remains a barrier to increasing diagnostic yields in genetic testing. At this point in time, few diagnostic clinical genetics laboratories in the UK perform any splicing analysis at the level of RNA, and there are no clear guidelines on how to interpret genomic variants potentially affecting splicing.

In addition to lack of confidence in classifying intronic variants as altering splicing, it is likely that many exonic variants affecting splicing are misclassified as missense or non-pathogenic synonymous changes. An estimated 22-25% of exonic variants classified as missense or nonsense actually affect splicing [117, 118]. For example, compound heterozygous variants in *LZTFL1* (BBS17) predicted to introduce missense changes were actually found to produce truncated protein (35kDa instead of 30kDa), suggesting that these variants actually impact on splicing [119]. A predicted missense variant at the end of exon 9 of *CSPP1*, a gene which is associated with a range of severe ciliopathies, was found to abolish normal splicing, leading to inclusion of an additional 10bp of sequence, shifting the reading frame and introducing a premature stop codon [78]. A predicted missense variant at the end of exon 4 of *TMEM231* was also shown to be affecting splicing and causing disease in MKS [120]. A single base pair substitution at the start of exon 16 in *DNAI1*, predicted to cause a missense amino acid change, was also shown to affect splicing, leading to skipping of exons 15 and 16 in patients with PCD [107].

It remains challenging to accurately predict the pathogenicity of variants potentially affecting splicing. The best optimisation of *in silico* splice prediction tools to date (using a combination of MaxEntScan with a 15% cut-off value and the PWM model with a 5% cut-off value in a study of *BRCA1* and *BRCA2* variants) achieved a sensitivity of 96% and specificity of 83% [121]. In practice, the accuracy of *in silico* splice prediction tools is often significantly lower. The contribution of coding sequence variants affecting splicing (such as synonymous mutations affecting exonic splicing enhancers) and deep intronic changes affecting splicing are particularly likely to be underestimated as they are often filtered out in genetic analysis pipelines which focus on non-synonymous coding and splice site variants. Furthermore, exome sequencing and gene panel sequencing often do not provide sequencing coverage of intronic variants further than 10-20bp from the intron/exon junction. As whole genome sequencing is more routinely used for disease diagnostics, thanks in part to the UK's 100,000 Genomes Project, more deep intronic variants are being revealed as causes of disease. For example, four new deep intronic variants in *USH2A* have been described in recent years [122, 123].

Diagnosing ciliopathies using RNA sequence analysis

Whilst *in silico* tools can be used to predict the effect of genomic DNA sequence variants on splicing, the most reliable method for identifying splicing changes is through direct RNA sequence analysis. Traditionally this has involved targeted RT-PCR of regions identified as potentially pathogenic from genomic DNA analysis, using RNA from relevant patient tissues or using minigene assays. There are many examples where RT-PCR of patient RNA has been employed to diagnose ciliopathies across the phenotypic spectrum including, but not limited to ; a deep intronic mutation in *OFD1* as a cause of XL-RP [124]; an intron 3/exon 4 splice acceptor site mutation in *BBS2* as a cause of BBS [105]; c.505+2T>C in *B9D1* as a cause of MKS [125]; two different splice-altering variants in *CEP78* as a cause of USH [126]; three different splice-altering mutations in *DNAI1* as a cause of PCD and; a splice acceptor site mutation in *TCTN3* as a cause of JBTS [84]. Minigene assays have been used to confirm splicing defects as causes of disease in various ciliopathies, including; various splice mutations in *BBS2*, *BBS3*, *BBS4* and *ALMS1* causing BBS [127] and; c.3112-5T>A in *IFT127* in patients with isolated RP [128]. However, whilst minigene assays give some insight into the effect of mutations on splicing, they may not be truly representative of the exact splicing defect *in vivo*. In the case of this variant in *IFT172*, the minigene assay showed that this genetic variant led to intron 28 retention, whereas RNA extracted from patient lymphoblastoid cell lines showed differential usage of the 3' splice site rather than complete intron retention [128]. Massively parallel whole transcriptome RNA sequencing (RNAseq) is a powerful technology which has the potential to revolutionise splice analysis, either in combination with genomic DNA sequence analysis, or as a standalone method. It has the potential to enhance diagnostic yields through direct detection of splicing aberrations caused by pathogenic variants, without the requisite prior knowledge for targeting RT-PCR. As a relatively new technology, there is little consensus on usage, interpretation, reliability or best practices for transcript-level analysis in RNAseq datasets, particularly for the purposes of novel transcript or novel splicing event identification, which is critical for the successful application of RNAseq to disease diagnostics.

One of the major fundamental challenges of analysing RNAseq data is accurately assembling long transcripts from short reads of sequence. Furthermore, estimation of isoform abundance from short sequence reads is statistically challenging, as each read samples only a small part of the transcript, and alternative transcripts often have substantial overlap. Mapping of reads to exons can also be challenging when there is differential 3' and 5' splice site usage. In the case of detecting novel splice events, particularly rare, disease specific events, or those which may be undergoing nonsense mediated decay (NMD), managing the balance between sensitivity and specificity is vital, to avoid overlooking disease causing events or having true events be swamped by false positives. This presents a major challenge for diagnosis of ciliopathies associated with genes with large transcript diversity, such as *IFT122*, associated with a form of SRPS. *IFT122* has at least four differentially spliced transcripts. One reported exon 6/intron 6 splice donor site mutation c.502+5G>A in *IFT122*, which causes exon 6 skipping, has been shown to only affect transcript isoform 3 (which includes exon 6), but not transcript isoform 4 (which does not include exon 6) [129]. Difficulty in differentiating between different transcripts using RNAseq could compromise the ability to detect pathogenic mutations such as this. In a separate case, amplification of cDNA from RNA from a parent carrying a splice-site variant in *TMEM231* was shown to display complete loss of heterozygosity of the disease allele, due to complete NMD of the aberrant transcript [120]. This highlights the potential challenges of capturing and identifying disease-associated RNA transcripts for the purposes of ciliopathy diagnostics. A further challenge stems from the tissue-specific nature of the expression and splicing of many ciliopathy

genes, in tissues which are not easily accessible and/or frequently biopsied during the course of care (e.g. blood, fibroblasts, muscle tissue). For example, only two of the recognised transcript isoforms of *IFT122* are expressed in blood, and any splice variant affecting other transcripts would not be detected by RNAseq performed on whole blood [129]. To address this issue, studies are increasingly expanding to extract RNA from more diverse patient materials, including skin biopsies/fibroblasts and urine-derived renal epithelial cells (URECs), which provides ciliated patient cells in a non-invasive manner [130]. This has been successfully applied in identifying variants causing exon 15 skipping in *NPHP3* in URECs. RT-PCR revealed wild-type (WT) mRNA from URECs harboured only transcripts containing exon 15, while heterozygous variant-carrying URECs from the patient's father showed an additional transcript with this exon being spliced out. In mRNA from blood, exon 15 is spliced out in healthy controls, which would likely have masked the variant's effect had this been the tissue of choice [109]. However, the success of this approach depends on robust transcriptional data from these tissues from healthy controls, and this data is lacking for many disease-relavant tissues. For example, in the study and diagnosis of PCD, multiciliated airway cells are obtained from patients through nasal brushings, which can be sampled fresh or grown at air-liquid interface (ALI) to generate a larger bank of patient material [131], although ALI cultures show different phenotypes from fresh samples [132]. These samples are currently used for diagnostic imaging, but work is ongoing by our groups and others to characterise the transcriptome of control samples in order to permit RNA sequencing of patient samples to enhance genetic diagnosis of PCD. The issue of tissue-specific transcript expression is a particular challenge in diagnosis of retinal ciliopathies. As previously discussed, the retina, and photoreceptor cells in particular, exhibit highly tissue-specific transcriptional profiles, with many ciliopathy genes producing exclusively photoreceptor-specific isoforms which are specifically affected by disease-causing splice mutations which cannot be confirmed by RNA analysis from other tissues. Advances in retinal organoid culture techniques from induced pluripotent stem cells derived from patient fibroblasts have provided one solution to this problem, but this is an extremely laborious and time-consuming process, taking several months for retinal organoid cultures to develop mature photoreceptors [133]. Culture techniques are being accelerated [134], refined towards a standardised approach, and the transcriptional profile of these organoids being defined [135], including using single cell RNAseq to understand the transcriptional profile of different cell types of these retinal organoids [136]. As a result, retinal organoids are providing useful models for studying mechanisms of retinal disease [51] and effectiveness of novel molecular therapies [137] and diagnostics; they have recently been used to characterise the effect of a novel splice acceptor site variant in *USH2A* as a cause of RP [138].

Dozens of computational methods for RNAseq analysis have been developed, each with their own individual strengths and limitations. A comprehensive discussion of all available computational approaches is beyond the scope of this paper (see e.g. [139-141]), and we will focus only on a basic discussion of those that have been applied to splicing analysis for rare disease diagnostics to date. Four recent papers have been published exploring the application of RNAseq for diagnostic purposes in various Mendelian diseases. Cummings *et al*. and Gonorazky *et al*. both focussed on neuromuscular disorders, obtaining diagnostic yields of 35-36% from RNAseq on biopsied muscle tissue [142, 143]. The methodology utilised involved alignment using STAR's two-pass method [144], followed by filtering and prioritisation of splice junctions of potential diagnostic relevance using code developed by Cummings *et al.*, along with investigating allelic imbalance in expression. Kremer *et al*.'s focus was mitochondrial disorders, with RNAseq performed on patient derived fibroblasts [145]. Again, STAR

was the alignment method of choice, coupled with LeafCutter[146] for detection and prioritisation of aberrant splicing events, and investigation of aberrant and monoallelic expression was done, achieving a diagnostic result in 10% of patients. Finally, Frésard *et al.* used RNAseq on blood mRNA in the diagnosis of a cohort of patients with a diverse range of rare diseases spanning 11 categories, including neurology, hematology and ophthalmology [147]. Using a combination of techniques including detection of expression and splicing outliers, a diagnostic rate of 8.5% was obtained. Candidate diagnoses were also identified for several other patients, including those with neurological phenotypes, for whom blood would not be an obvious tissue of interest, demonstrating the potential for wider applicability, albeit with a lower diagnostic yield.

Common themes in the analysis strategies in the above papers include the use of STAR for alignment, and the leveraging of publically available RNAseq data (e.g. GTEx https://gtexportal.org/) as controls. Despite many different alignment tools being available, STAR appears to be emerging as a front-runner for RNAseq, likely due to its speed and ease of use, clear documentation and active support. STAR in two-pass mode utilises known splice junctions from a provided transcriptome, identifying potential novel junctions well supported by split reads (those spanning a splice junction) in the data on the first pass, then re-traverses the data to provide read counts for all junctions[144]. Utilising publically available RNAseq data, which most of the above papers do, is a powerful strategy for reducing noise and removing from analysis splice junctions that are present in non-diseased individuals and are thus unlikely to be the aberrant splicing events responsible for disease in the patient in question. Careful matching of tissue type and study design, and reprocessing of control data alongside cases can help ensure maximum consistency between different datasets.

A further issue to resolve to optimise the use of transcriptomics for rare disease diagnostics is the depth of sequencing required to achieve robust detection of rare splice forms. At low sequencing depth, RNA sequencing experiments likely fail to detect the majority of low-abundance transcripts, which only become apparent through deep RNA sequencing. Typical differential gene expression analysis studies sequence to a depth of 20 million reads per sample. In the retina, only approximately 50% of exons will be covered with sequencing at this depth [25]. For splicing analysis, read depths of 50-100 million reads per sample have typically been employed [142, 143], as greater depths are required to detect rare, patient specific splicing events, and those that may be undergoing NMD.

As more research is undertaken using RNAseq diagnostically, larger and more diverse datasets will facilitate the development of optimal methods both in terms of study design and analytical practice. Responsible sharing of data will be crucial in maximising both broad scientific and diagnostic benefit. In the UK, the NIHR have recently funded a program of research into clincial diagnostic uplift in a range of genetic disorders, including PCD, where best practice guidelines for the effective utilisation of RNAseq in a diagnostic setting will be explored.

Treating ciliopathies by targeting splicing

Ciliopathies have long been considered untreatable and incurable conditions. Recent advances in gene therapy have challenged this view. Early efforts have focussed on gene delivery by viral vectors for treatment of the most common causes of recessive retinal ciliopathies. This has led to two

independent clinical trials for treatment of the RP phenotype in USH patients carrying *MYO7A* mutations (https://clinicaltrials.gov/ct2/show/NCT02065011; https://clinicaltrials.gov/ct2/show/NCT01505062), and two independent Phase1/2 clinical trials for treatment of XL-RP associated with mutations in *RPGR* (https://clinicaltrials.gov/ct2/show/NCT03316560; https://clinicaltrials.gov/ct2/show/NCT03252847?cond=Retinitis+Pigmentosa&rank=38). Excitingly, a third *RPGR* gene augmentation trial is now recruiting for Phase2/3 (https://clinicaltrials.gov/ct2/show/NCT03116113). *RPGR* mutations are responsible for 70-90% of XL-RP cases, and 10-20% of all RP cases [40]. Mutations in *MYO7A* account for around 20% of USH cases [148]. However, some of the most common genetic causes of ciliopathies are very large genes which cannot be delivered by viral vectors. Adeno-associated virus serotype 2 (AAV2), the commonly used viral vector, is limited to delivery of around 3.5kb of genetic material, including promoter and polyadenylation sequence [149]. *USH2A*, which accounts for 10 to 15% of cases of AR-RP and 50% of USH cases, has a 12kb coding sequence. The most common genetic cause of ciliopathy, *PKD1*, which accounts for 85% of cases of ADPKD, has a coding region of almost 13kb.

As a result, alternative approaches are now under investigation, many of which focus on altering splicing. Antisense oligonucleotides (ASOs) are one technology which can be used to modulate splicing. ASOs are short, single-stranded synthetic oligodeoxynucleotides which are designed to target specific regions of mRNA to modulate splicing to correct genetic defects causing disease. They can be used to promote retention of exons that are otherwise skipped in affected patients, and skip pseudoexons which are otherwise retained in affected patients [150]. ASOs can also be used to induce skipping of exons carrying premature stop codons or insertions/deletions causing a frameshift, to restore the reading frame and restore protein production. Indeed, this approach has led to the first FDA-approved ASO for treatment of Duchenne Muscular Dystrophy (DMD), eteplirsen [151] and the first FDA-approved ASO for treatment of Spinal Muscular Atrophy (SMA), nusinersen [152].Eteplirsen is an ASO designed to induce exon 51 skipping of *DMD* pre-mRNA, which encodes dystrophin, which is mutated in patients with DMD [153]. Eteplirsen causes exon 51 skipping which restores the reading frame of *DMD* in DMD patients with out-of-frame deletions in the *DMD* gene, restoring dystrophin protein production [154, 155]. The protein produced is truncated, but this is sufficient to reduce the severity of patient phenotype, slowing respiratory decline [156] and extending the number of years a patient remains ambulatory compared to untreated identical twin patients [157]. Nusinersen is an ASO designed to promote exon 7 inclusion in mature *SMN2* mRNA through blocking the binding site of hnRNP in intron 7 of *SMN2* pre-mRNA[158, 159]. This hnRNP binding site acts as a splicing silencer in patients with SMA, resulting in exclusion of exon 7 and truncated protein production. Nusinersen restores full-length protein production and improves motor function and probability of survival in treated early-onset SMA patients compared to sham control [152, 160]. It is also successful in improving motor function in later-onset SMA patients [161].

ASO technology has improved in recent years to reduce off-target effects and toxicity, improve nuclease resistance and target-binding affinity [162]. This has been achieved through modification of the ASO backbone such as 2' ribose modifications e.g. 2'-*O*-methylation (OMe), 2'-O-methoxy-ethylation (MOE), and locked nucleic acid (LNA), and P backbone modifications such as phosphorothioate and morpholino [163, 164] (**Figure 3**). Conjugation of the backbone to a carrier or ligand, such as lipids, peptides, carbohydrates or antibodies, has helped to improve bioavailablity and

delivery across membranes, and tissue-specific delivery [165, 166]. These improvements are making successful clinical applications more likely. For example, FDA-approved eteplirsen is a phosphorodiamidate morpholino oligonucleotide [151, 153, 154] and nusinersen is a 2′-O-(2-methoxyethyl) modified ASO.

The ASO treatment approach is particularly useful in conditions associated with a common mutation which affects splicing. There are several common founder mutations in *USH2A*, including deep intronic c.7595-2144A>G and which introduces a novel splice donor site in intron 40, leading to inclusion of a novel exon (PE40) in the mature transcript [123]. A recent study showed successful specific induction of exon skipping of this PE40 in patient fibroblasts and in a minigene assay [98]. A first-in-man study of the safety and tolerability of RNA ASO therapy for RP and USH patients with mutations in exon 13 of *USH2A* is currently underway, coordinated by the University of Michigan Kellogg Eye Centre and Retina Foundation of the Southwest, Dallas (https://clinicaltrials.gov/ct2/show/NCT03780257). This study is testing QR-421a, which targets exon 13 to induce exon 13 skipping in patients with nonsense or frame-shift mutations in exon 13 of *USH2A*, including the common c.2299delG and the c.2276G>T mutations (https://databases.lovd.nl/shared/variants/USH2A/). This aims to restore the reading from of *USH2A* mRNA and restore functional usherin protein production in the retina of patients to prevent retinal degeneration and vision loss. Another common mutation which affects splicing in ciliopathy patients, is the deep intronic variant c.2991+1655A>G (sometimes called IVS26) in *CEP290* which accounts for 20-25% of cases of LCA [93]. The change introduces a novel splice donor site and insertion of a novel cryptic exon, exon X, in the transcript, creating a premature truncation codon p.(Cys998*), probably subjecting the transcript to NMD (**Figure 5a**). A proof-of-principle study in 2012 showed the efficacy of an ASO approach in correcting *CEP290* splicing in human patient fibroblasts by blocking exon X to exclude it from the final transcript and restore WT transcript production [167]. A recent study has shown success of clinical drug candidate ASO QR-110, a single-stranded, fully phosphorothioated, and 2′ *O*-methyl-modified RNA oligonucleotide (**Figure 3**), in rescuing the *CEP290* splicing defect by blocking exon X splicing to restore WT CEP20 mRNA production in pre-clinical models including human patient-derived retinal organoids [137]. QR-110 was the most successful of 29 ASOs designed to target the region of exon X using an 'oligo-walk' approach [137]. This led to a phase 1/2 clinical trial of intravitreal injection of QR-110, (https://clinicaltrials.gov/ct2/show/NCT03140969) which showed safety and tolerability, and an exceptional improvement in vision in one patient [168]. This is now entering phase 2/3 clinical trials (https://clinicaltrials.gov/ct2/show/NCT03913143).

Whilst initial efforts have focussed on treating the retina, as it is a relatively small area to treat and relatively minor improvements in cell functionality can lead to significant clinical outcomes for patients, work is now expanding into exploring systemic treatment of syndromic ciliopathies. In the first study of its kind worldwide, Prof John Sayer and colleagues showed effectiveness of ASO-induced splicing of a mutated exon (41, G1890*) of *CEP290* restoring CEP290 protein expression in kidney cells of JBTS patients. In treated cells, protein localises correctly to cilia and restores normal cilium length, whereas patient cilia overgrow in length. Systemic treatment in a Cep290 mouse model led to reduced cystic burden in the kidney [169].

Gene editing approaches are also being trialled as methods for correcting pathogenic splice variants in ciliopathy patients. This has the advantage of stably and permanently correcting the genome of patients. A recent study reported successful editing of the common intronic mutation in *CEP290* in

humanised CEP290 mutant mice. Adeno associated virus 5 (AAV5) was used to deliver two guide RNAs and *Staphyllococcus aureus* Cas9 to delete or invert the region of exon 15 containing the common intronic mutation in *CEP290* [170]. Sub-retinal injection of this therapeutic, named EDIT-101, into humanised CEP290 mutant mice resulted in efficient and specific CEP290 gene editing, but it is unclear whether this led to clinically significant improvements in visual function. EDIT-101 is now entering phase 1/2 clinical trials (https://clinicaltrials.gov/ct2/show/NCT03872479).

Spliceosome-mediated RNA *Trans*-splicing (SMaRT) [171, 172] is an alternative approach to rescuing splicing defects in ciliopathy patients. This is a modified gene delivery approach which involves delivering a partial coding DNA sequence (a 'pre-mRNA trans-splicing molecule' or PTM) designed to *trans*-splice to endogenous pre-mRNA. A single PTM is delivered by a viral vector to target endogenous pre-mRNA transcripts to yield a hybrid exogenous-endogenous mRNA which either excludes a pathogenic cryptic exon, an exon carrying a stop or frameshift mutation, or includes a skipped exon. This represents a safer approach than gene delivery which can result in toxic levels of overexpressed protein, or gene editing with dangers of off-target nuclease activity. A further advantage is that this approach can correct a larger range of mutations with one single therapeutic, which may be particularly helpful for patients with compound heterozygous mutations. A recent study has shown the effectiveness of this approach in correcting the mis-splicing caused by *CEP290* c.2991+1655A>G *in vitro* and *in vivo* in a mouse minigene model of *CEP290* intron 26-27 (**Figure 4**) [173]. In this study, the PTM includes a CMV promotor, a codon optimised partial coding sequence (PCDS) of *CEP290* cDNA from position 1 (A of ATG) to 2991, a 5' splice site and a sequence complementary to intron 26-27 of CEP290, upstream of c.2991+1655A>G, and a polyA tail to aid entry into the spliceosome. When transfected into cells, this minigene is expressed to produce an RNA molecule consisting of the PCDS and a binding domain which complementarily binds to endogenous *CEP290* pre-mRNA upstream of the c.2991+1655A>G mutation (**Figure 4b**). The RNA product from the minigene and the endogenous *CEP290* pre-mRNA then undergo *trans*-splicing in the spliceosome to produce a hybrid *CEP290* mRNA which includes minigene-derived PCDS sequence from exons 1-26 and endogenous sequence from exon 27-54, with exon X excluded (bottom schematic in **Figure 4c**) which encodes full-length wild-type CEP290 protein. Thus, trans-splicing bypasses the effect of the c.2991+1655A>G mutation and restores full-length wild-type CEP290 protein production [173].

Delivery of modified U1 snRNA to cells affected by a pathogenic splicing change can also be used to correct splice defects. U1 snRNA binds the splice donor site to initiate exon recognition during splicing, and delivery of a U1 snRNA optimised with increased binding affinity to a mutant splice donor site can repair splicing. Aberrant splicing of exon 5 of *BBS1*, associated with a splice donor site mutation, was partially corrected in patient fibroblasts by lentiviral delivery of a sequence-modified synthetic U1 snRNA in a dose-dependent manner [174]. Similarly, this therapeutic U1 snRNA approach has been used to correct mis-splicing of *RPGR* in patient fibroblasts with point mutation in exon 10 [174, 175]. This is a promising strategy for treatment of patients with splice mutations in *BBS1* or RPGR, the most common genetic cause of BBS and XL- RP respectively. However, these have not progressed towards gene therapy as of yet.

Conclusion and future perspective

Splicing contributes significantly to the pathogenesis, diagnosis and future treatment strategies of ciliopathies. Pathogenic splice variants have been reported in more than half of the known ciliopathy

genes (**Table 1**), and there is probably an underestimate of the contribution of splicing defects to ciliopathies due to difficulty in deciding whether a 'splice variant' is pathogenic. When we reviewed pathogenic splice variants in ciliopathy genes reported in the literature (as collated in HGMD, Stenson et al., 2003) we found that on average around 17.5% of variants affect splicing. However, when we compared this to variants in clinical variant interpretation database ClinVar (Landrum et al., 2014), only 6% of pathogenic variants affected splicing (**Table 1**). Many more variants affecting splicing were assigned 'uncertain significance' status. It seems clear that there remains uncertainty when assigning pathogenic status to splice variants, and this remains a barrier to increasing diagnostic yields in genetic testing. At this point in time, few diagnostic clinical genetics laboratories in the UK perform any splicing analysis at the level of RNA, with no commissioned clinical service and no clear guidelines on how to interpret genomic variants potentially affecting splicing. At this critical time, and with the advent of the integration of next generation sequencing into clinical practice, it is important to incorporate clinical RNA analyses to maximise diagnostic uplift for patient benefit. Indeed for some disorders this may mean RNA first and DNA second. For ciliopathies in particular we can achieve this by building a robust body of knowledge of the control of the transcriptome and how this changes in disease - a form of 'biomarker'. Development of both the transcriptome methods and pipelines of analyses as well as clear guidelines for interpretation will be essential. Finally, as we improve our understanding of how sequence changes affecting splicing cause disease, we will better understand the complex mechanism of splicing, facilitating development of innovative methods for manipulation of the splicing process, revealing future therapeutic targets.

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Figure and Table legends

Figure 1: Schematic representation of primary cilium, motile cilia and photoreceptor cilium
(**a**) Schematic figure of a typical epithelial cell with single apical non-motile primary cilium. Cilium membrane in orange, microtubule doublet axoneme and triplet basal body in red. (**b**) Cross-section of axoneme of non-motile primary cilium showing radial arrangement of microtubule doublets in red. (**c**) Schematic figure of a rod photoreceptor cell with highly modified cilium. Cilium membrane in orange, microtubule doublet axoneme and triplet basal body in red, membrane stacks with rhodopsin molecules as purple dots. (**d**) Cross-section of axoneme of photoreceptor cilium showing radial arrangement of microtubule doublets in red. (**e**) Schematic figure of a typical epithelial cell with multiple apical motile cilia. Cilium membrane in orange, microtubule doublet axoneme in red. (**f**) Cross-section of axoneme of non-motile primary cilium showing radial arrangement of microtubule doublets plus central pair of microtubules in red, nexin links in navy, radial spokes in purple, and inner and outer dynein arms in green.

Figure 2: Typical clinical features of ciliopathies
(a-c) Brain MRI findings for an individual with Joubert syndrome, showing the molar tooth sign with moderate vermis hypoplasia, elevated and thickened superior cerebellar peduncles (arrowhead), and superior cerebellar dysplasia (arrow) indicated. Reproduced from Wheway et al., 2015. **(d, e)** Clinical features of individuals with a short-rib thoracic dystrophy, including narrow and deformed thorax. Reproduced from Wheway et al., 2015. (**f**) Clinical pictures of a male Alstrom syndrome patient at age 6 years 8 months, presenting with truncal obesity. Note characteristic face and prominent ears. Reproduced from Marshall et al., 2011 under the terms of the Creative Commons Attribution License CC BY. **(g)** Massive swelling of the abdomen of a foetus at gestation age 18+/40 with Meckel-Gruber

syndrome due to grossly enlarged, cystic kidneys. Reproduced from Hartill et al., 2017 under the terms of the Creative Commons Attribution License CC BY. **(h)** Cystic dysplasia of the kidneys comprising large, fluid-filled cysts, small cysts and cystic swelling of the proximal tubules and glomeruli, with absence of normal renal parenchyma in MKS. Reproduced from Hartill et al., 2017 under the terms of the Creative Commons Attribution License CC BY. (i, j) postaxial polydactyly on both hands of a patient with orofaciodigital syndrome. Reproduced with permission from Bonnard et al., 2018. (**k, l**) Examples of fundoscopy images revealing mild pigmentary depositions and some mottling in syndromic ciliopathy patients with retinal dystophy. Reproduced from Wheway et al., 2015. (**m**) coronal computed tomography (CT) scan of a 17-year-old PCD patient showing diffuse sinusitis with mucosal thickening and polyposis. **(n)** Endoscopic view showing nasal polyp in a PCD patient **(o)** Chest X-ray of a 6-year-old PCD individual with middle lobe atelectasis. Silhouetting of the right heart border is present. **(p)** Chest CT scan of a 6-year-old individual with *situs inversus totalis*. The left-sided middle lobe shows extensive bronchiectasis with volume loss (white arrowhead). In addition, consolidations and mucous impaction are present in the right upper lobe. Reproduced from Werner et al., 2015 under the terms of the Creative Commons Attribution License CC BY.

Figure 3. Oligonucleotide backbones for antisense oligonucleotide therapeutics

The figure shows basic chemical structure of an unmodified DNA or RNA molecule, and modified structures (with modification highlighted with pink circle) used for antisense oligonucleotide therapeutic treatment of ciliopathies.

Figure 5. Strategy to rescue aberrant *CEP290* splicing through spliceosome-mediated 5′ Pre-mRNA *trans*-Splicing

(**a**) Diagram of the most prevalent mutation in Leber congenital amaurosis type 10 c.2991+1655A>G, which introduces a new intronic canonical 5′ splice site (5′ SS). The novel splice site leads to inclusion of a cryptic exon, exon X. This cryptic exon encodes a premature stop codon (black octagon) leading to a truncated protein. (**b**) Schematic of an approach to utilize a 5′ pre-mRNA *trans*-splicing molecule (PTM) to rescue mutations in *CEP290* that are located 5′ to intron X-27. The PTM transcript consists of the partial coding DNA sequence (PCDS) encoding CEP290 exons 1-26, the novel 5' SS, a spacer and a 'putative binding domain', which is reverse complementary to the target sequence in intron X-27. (**c**) Three potential splicing outcomes with *CEP290* c.2991+1655A>G following introduction of a 5′ PTM: (1) joining of exon 26 to exon 27 from *cis*-splicing for the wild-type junction; (2) inclusion of exon X from *cis*-splicing (predominant mRNA species with c.2991+1655A>G present); (3) joining of the 5′ PCDS to exon 27 from *trans*-splicing. Both outcomes 1 and 3 would result in full-length CEP290 peptide (because the PCDS is designed such that it encodes exons 1-26). Reproduced with modification from Dooley et al., 2018 under the terms of the Creative Commons Attribution License CC-BY.

Table 1. Class, OMIM phenotype number, disease genes and hallmark clinical features of major ciliopathies

Major ciliopathies, grouped into non-motile and motile, subdivided into neurodevelopmental, skeletal, obesity, sensorineural, retinal and respiratory. Names, abbreviations and OMIM phenotypes are given for each, alongside a list on currently known disease genes associated with each condition, and hallmark clinical features.

Table 2. Detailed phenotypes and types of mutation reported in known ciliopathy genes

List of known ciliopathy genes, OMIM gene number, ciliopathy(s) associated with this gene, types of mutation reported, and percentage of reported mutations which affect splicing according to HGMD (Stenson et al., 2003) and ClinVar. Half of all ciliopathy genes (104/188) have at least one reported splicing variant associated with disease. Of the genes in which splicing variants have been reported in the literature these account for on average 17% of total reported variants. Clinical interpretation of splicing variants would seem to underestimate the pathogenicity of such variants.

Table 3. Ciliopathy abbreviations, full names and incidence rates per 100,000 in the general population

Abbreviation of ciliopathy, full name and estimated prevalence, from OMIM, Orphanet Rare Disease or published literature

Primary cilia are essential signalling organelles found on the apical surface of epithelial cells, where they coordinate chemosensation, mechanosensation and light sensation. Motile cilia play a central role in establishing fluid flow in the respiratory tract, reproductive tract, brain ventricles and ear. Genetic defects affecting the structure or function of cilia can lead to a broad range of developmental and degenerative diseases known as ciliopathies.

Splicing contributes to the pathogenesis, diagnosis and treatment of ciliopathies. Tissue-specific alternative splicing contributes to the tissue-specific manifestation of ciliopathy phenotypes, for example the retinal-specific effects of some genetic defects, due to specific transcript expression in the highly specialised ciliated cells of the retina, the photoreceptor cells. Ciliopathies can arise both as a result of genetic variants in spliceosomal proteins, or as a result of variants affecting splicing of specific cilia genes. Here we discuss the opportunities and challenges in diagnosing ciliopathies using RNA sequence analysis and the potential for treating ciliopathies in a relatively mutation-neutral way by targeting splicing.

Splicing in the pathogenesis, diagnosis and treatment of ciliopathies

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Abstract

Primary cilia are essential signalling organelles found on the apical surface of epithelial cells, where they coordinate chemosensation, mechanosensation and light sensation. Motile cilia play a central role in establishing fluid flow in the respiratory tract, reproductive tract, brain ventricles and ear. Genetic defects affecting the structure or function of cilia can lead to a broad range of developmental and degenerative diseases known as ciliopathies.

Splicing contributes to the pathogenesis, diagnosis and treatment of ciliopathies. Tissue-specific alternative splicing contributes to the tissue-specific manifestation of ciliopathy phenotypes, for example the retinal-specific effects of some genetic defects, due to specific transcript expression in the highly specialised ciliated cells of the retina, the photoreceptor cells. Ciliopathies can arise both as a result of genetic variants in spliceosomal proteins, or as a result of variants affecting splicing of specific cilia genes. Here we discuss the opportunities and challenges in diagnosing ciliopathies using RNA sequence analysis and the potential for treating ciliopathies in a relatively mutation-neutral way by targeting splicing.

The cilium in inherited human disease

The primary cilium (**Figure 1a,b**) is the cell's central signalling organelle and is fundamentally important for normal development and physiology [1]. Cilia are found throughout the body on the apical surface of most epithelial cells, where they play roles in mechanosensation and chemosensation. A highly specialised primary cilium, the photoreceptor cilium (**Figure 1c,d**), is found on the photoreceptor cell of the retina. This photoreceptor cilium contains proteins which coordinate light sensation [2]. Motile cilia (**Figure 1e,f**), which have a different ultrastructure from primary cilia allowing them to beat and establish fluid flow, also play an essential role in development, in the establishment of left-right body asymmetry. At the embryonic node in early development, a mixed population of motile and non-motile cilia are found. The motile cilia establish leftward flow to move left-specific morphogens to the left-hand side of the developing embryo, defining this as the left. In developed mammals, motile ciilia play a crucial role in movement of mucus in the respiratory tract, ova along the Fallopian tubes, cerebrospinal fluid in the ventricles of the brain, and fluid in the middle ear [3].

When cilia do not form or function properly, a range of inherited conditions called ‘ciliopathies’ arise [4]. These are separated into the non-motile and motile ciliopathies. Due to the widespread importance of the primary cilium across multiple tissues, the non-motile ciliopathies tend to be syndromic conditions, affecting multiple organs. Common features of syndromic non-motile ciliopathies are neurodevelopmental defects (**Figure 2a-c**), skeletal defects (**Figure 2d,e**), obesity (**Figure 2f**), cystic kidney disease (**Figure 2g,h**), and inner ear problems leading to sensorineural hearing loss. Polydactyly (**Figure 2i,j**) and retinal dystrophy (**Figure 2k,l**) are particularly common across syndromic ciliopathies. The non-motile ciliopathies can be broadly classified into the

neurodevelopmental ciliopathies, such as Joubert (JBTS) and Meckel-Gruber syndrome (MKS); the kidney ciliopathies such as polycystic kidney disease (PKD) and nephronophthisis (NPHP); the skeletal ciliopathies such as short-rib thoracic dystrophies (SRTD) and orofaciodigital syndrome (OFD); the obesity ciliopathies such as Bardet-Biedl syndrome (BBS) and Alstrom syndrome (ALMS) and the isolated retinal ciliopathies, including subtypes of retinitis pigmentosa (RP), Leber congenital amaurosis (LCA) and cone-rod dystrophy (CORD) (**Table 1**). Defects in motile cilia lead to a group of disorders termed primary ciliary dyskinesia (PCD) (**Table 1**). PCD is a clinically heterogeneous group of disorders which may include recurrent respiratory infections, progressive upper respiratory problems and loss of lung function, subfertility, infrequent hydrocephalus, situs defects (which may be linked to heart disease) and hearing problems (**Figure 2m-p**) [5].

The ciliopathies are widely genetically heterogeneous (**Table 2**). The vast majority of these conditions are inherited in an autosomal recessive manner, but there are also examples of X-linked inheritance such as X-linked JBTS and X-linked oro-facio-digital syndrome (OFD) associated with *OFD1* mutations. However, the single most common ciliopathy is autosomal dominant PKD (AD-PKD), 85% of which is caused by mutations in *PKD1* [6] (**Table 3**). AD-PKD is the most common cause of end-stage renal failure, and affects between 1:1000 and 1:4000 individuals in the EU, making it one of the most common genetic diseases in humans, and the most common cause of end–stage renal failure [7]. Non-syndromic retinal ciliopathies are also relatively common. Retinal dystrophies collectively affect around 1:3000 people worldwide [8]. The most common of these is RP, which affects around 1:3500 people worldwide [9]. LCA affects around 1:50,000 people and CORD affects around 1:40,000 people worldwide. Around one third of the genetic causes of retinal dystrophy encode proteins of the photoreceptor cilium [10]; **Table 2**), so non-syndromic retinal ciliopathies can be estimated to affect 1:6000 individuals in the general population worldwide (**Table 3**). The recessive ciliopathies are individually much rarer but collectively common, with an incidence rate from 1 in 15-30,000 (USH) to 1 in 100,000 (JBTS, BBS, MKS and ALMS) worldwide (**Table 3**). PCD affects around 1 in 10,000 people worldwide (**Table 3**) and is genetically heterogeneous, with 38 known genetic causes [11] (**Table 2**). As with non-motile ciliopathies, the majority of genetic subtypes of PCD are inherited in an autosomal recessive manner, but X-linked forms caused by mutations in the *RPGR* or *PIH1D3* genes have been reported. Collectively, ciliopathies can be estimated to affect between roughly 1:700 and 1:2000 people in the general population worldwide [12] (**Table 3**).

The importance of alternative splicing in ciliated cells

The vast majority of human genes contain more than one exon, divided by introns, and splicing is the process by which introns are removed from a pre-mRNA and exons joined to make a mature mRNA. This process is catalysed by a large protein and RNA complex known as the spliceosome [13]. The spliceosome is composed of 5 small nuclear RNAs (snRNAs), U1-U5, and many proteins, together making 5 snRNPs. In the process of splicing, U1snRNP recognises and binds the splice donor site (the 5' splice site), and promotes the binding of U2snRNP to the branch site. Independently of this, the U4/U6.U5 tri-snRNP forms in the cell, and is recruited to the pre-mRNA, where U6snRNP replaces U1snRNP. This forms the catalytically active spliceosome, which excises the intron and joins the exons through two transesterification reactions [13]. Alternative splicing is a process central to the functioning of all human cells, with around 95% of multiexon genes undergoing alternative splicing [14, 15]. Alternative splicing involves differential use of 5' (splice donor) and 3' (splice acceptor) splice sites, differential inclusion or exclusion of exons (including mutually exclusive exons) and differential

intron retention/exclusion [15]. Alternative splicing controls fundamental cell processes including protein transport across membranes [16, 17]. Differential transcript expression and alternative splicing is highly tissue-specific. Indeed, patterns are more similar between the same tissues of different species than they are between different tissues of the same organism [18]. Such differential splicing drives different developmental processes across different organs and tissues [19] and contributes to the specific functions of mature cells such as neurons [20, 21]. Ciliated cells are no different from other cells in this regard. However, one particular ciliated tissue deserves special consideration here, for the complexity of its splicing profile; the retina.

Gene expression is extremely heterogeneous early in eye development, which begins at 3.5 weeks post conception (PCW) and continues until 5 months after birth [22]. According to gene expression patterns, there are 3 broad phases of eye development; developing eyes from 4.6-7.2 PCW; developing retinae from 7.7-10 PCW; and developing retinae from 12-18 PCW [22]. Peak expression of genes involved in connecting cilium and photoreceptor outer segment biogenesis occur from 12-18 PCW [22]. The photoreceptor cells in particular display a 'switch-like' splicing pattern, which involves a high rate of inclusion of exons which are not included in transcripts in any other cell type of the body [23]. Whilst photoreceptor cells are sensory neurons, their splicing pattern is not controlled by typical sensory neuron splicing factors, but rather by Musashi 1 (MSI1) protein [23]. Before photoreceptor outer segment development, there is a high rate of specific splicing of primary cilia genes, suggesting that the splicing of these directs the development of the outer segment from a regular primary cilium [23]. Patterns of splicing are highly dynamic through eye development, with variable rates of retained introns, skipped exons, alternative 3′ splice sites, alternative 5′ splice sites and mutually exclusive exons seen in transcripts at different stages of eye development. At 12-18 PCW, significantly different splicing of ciliary genes and genes involved in the RNA splicing process itself is seen in retina, compared to 7.7-10 PCW [22]. This suggests that alternative splicing is particularly important for regulation of the splicing process itself from 7.7-10 PCW, and for regulation of connecting cilium and outer segment biogenesis, from 12-18 PCW. The observation that genes involved in pre-mRNA splicing are significantly differentially spliced at 7.7-10 PCW was confirmed in analysis of similar expression data deposited by an independent group [24]. The authors of this paper suggest that this window of retinal development (7.7-10 PCW) is the stage at which a new specific splicing programme is being established for the next developmental stage, and this explains the enrichment of differentially spliced pre-mRNA splicing genes at this stage of retinal development [22]. The adult human retina also displays extensive transcript diversity. Deep RNA sequencing (300 million reads per samples) of three human retinae identified 79,915 novel alternative splicing events, including 29,887 novel exons, 21,757 3' and 5' alternative splice sites, and 28,271 exon skipping events, and 116 potential novel genes [25]. A large expression study of 50 human retinae confirms the finding that around 50% of transcripts in the retina differ from the GENCODE reference transcriptome, including from 206 putative novel genes [26]. Transcript expression varies across anatomical locations in the retina, both in different cell types [27, 28], and different regions (nasal/temporal and central retina/peripheral) [29]. In particular, there is greater transcript diversity in the neural retina, with around 15,000 alternative splice events are seen in the neural retina, compared to around 10,000 in the retinal pigment epithelium (RPE), choroid and sclera [28]. This is in keeping with the general observation that neural tissues (and testes) exhibit higher transcriptome complexity than other tissues [30-32]. However, it is difficult to make a direct comparison of splicing complexity in the retina compared to other tissues, because the retina has historically been neglected from large expression studies such as GTEx [33] due to difficulty in

obtaining high quality human retinal samples. The physiological relevance of novel retinal genes and isoforms remains to be fully understood, but analysis of novel genes co-expressed with rhodopsin (*RHO*), followed by gene ontology enrichment analysis suggests roles in visual perception, photoreceptor outer segment membrane function, cilium structure and function [26]. Advances in human stem-cell derived retinal organoid culture (discussed later) provide a valuable experimental model for further studies into the function of specific retinal transcripts.

Many genes linked to human retinal disease are known to have retinal-specific isoforms. These genes are alternatively spliced in developing and mature retina, showing different patterns of splicing at different points of development [22]. *ARL6* (BBS3), which can be associated with BBS and non-syndromic retinal dystrophy, has a retina-specific splice variant BBS3L which includes an extra 13bp coding exon near the 3' end (cassette exon). This shifts the reading frame, removes 7 amino acids from the C-terminus of the protein and adds 15 novel amino acids in their place [34]. *MAK* encodes a transcript which includes exon 12 (cassette exon) which is only expressed in the retina and *CEP78* has three retinal isoforms, which include cassette exons 15 and 17 [35]. *RPGRIP1* has a novel retinal transcript which uses an alternative 5' splice site for exon 13 and skips 33 nucleotides from the 5' end of canonical exon 13. This isoform including exon 13d is also expressed at a low level in liver, pancreas and placenta [36]. *TTC8*, which is mutated in BBS type 8, has a retinal-specific isoform which includes a cassette exon, named exon 2a, which was not identified until studies were undertaken in patients with RP, who were found to have -2 splice acceptor site mutation in intron 1 of *BBS8* causing their disease [37]. Studies have confirmed that this splicing mutation, which specifically causes exon skipping of the retinal-specific cassette exon 2a, causes RP due to tissue-specific expression of this transcript exclusively in photoreceptor cells [38]. *BBS5*, which is mutated in BBS, has a retinal specific isoform in most vertebrates. In the mouse, this is formed from the use of cryptic splice sites in intron 7, producing a transcript which produces a truncated protein with a novel C-terminal end [39]. Of particular relevance to human disease is *RPGR*. Mutations in *RPGR* account for 70-90% of XL-RP cases, and 10-20% of all RP cases [40]. There are at least twelve different isoforms of *RPGR*, including two major retinal protein isoforms of RPGR; RPGR(1-19) and RPGR(1-ORF15) [41]. RPGR(1-ORF15) is a retina-specific protein isoform which includes a large 3' terminal exon (ORF15). The retinal-specific terminal exon ORF15 is mutated in 60% of XL-RP patients [42]. Most mutations are small deletions or nonsense mutations leading to premature termination of translation and truncation of the protein, but some affect splicing [42, 43]. Outside of ORF15, splicing mutations are more common than changes in the coding sequence [44, 45]. A +1G>T splice site mutation in intron 5 of *RPGR* has been reported in a family with RP and recurrent respiratory infections [46]. G>T transversion at nucleotide 1164 of intron 15 which may create donor splice site has been reported in one family with atrophic macular degeneration [47]. At the time of writing, 11 splice site variants in *RPGR* are deposited in the clinical variant database ClinVar [48], but only 1 is annotated as 'pathogenic'. This suggests that there is current difficulty in assigning pathogenic status to variants potentially affecting splicing, and splice variants in *RPGR* may be a more common cause of disease than is currently appreciated.

Splicing in the pathogenesis of ciliopathies - retinal ciliopathies associated with spliceosomal proteins

Mutations in genes encoding proteins involved in pre-mRNA splicing, including *PRPF3*, *PRPF4*, *PRPF6*, *PRPF8*, *PRPF31*, *SNRNP200*, *DHX38*, *CWC27* and *RP9*, are the second most common cause of autosomal dominant RP and a minor cause of autosomal recessive RP. 17 years since the identification of *PRPF8*

as a cause of RP, it remains unclear why mutations in pre-mRNA splicing factors cause a phenotype restricted to the retina. A whole genome siRNA knockdown screen for ciliogenesis regulators in a ciliated kidney cell line identified a novel specific role of pre-mRNA splicing factors *PRPF6*, *PRPF8* and *PRPF31* in ciliogenesis [49]. The same observation was made in an independent reverse genetic screen [50]. Further investigation showed that these proteins localise to the base of the photoreceptor cilium, suggesting that these proteins have a role beyond splicing, and classifying these conditions as retinal ciliopathies [49].

Further study in retinal organoids and RPE derived from iPSCs from patients with *PRPF31* mutations show decreased efficiency of splicing in an E1A minigene reporter assay [51]. RPE from patient iPSCs also show a substantial downregulation of SART1, a U5 snRNP protein important for the formation of the pre-catalytic spliceosomal B complex, but no changes in the expression of the U5 protein PRPF8 or the U4/U6 protein PRPF4. Retinal organoids from patients showed differential expression of actin cytoskeleton, ciliary membrane, primary cilium, photoreceptor inner and outer segment, axon terminal and phototransduction proteins. In terms of differential splicing, retinal organoids from patients with *PRPF31* mutations showed an enrichment of mis-spliced centriole and microtubule organisation genes, with skipped exons, retained introns, alternative 5′ and 3′ splice sites, and mutually exclusive exons. In both RPE and retinal organoids derived from *PRPF31*$^{-/-}$ patients, the most significantly mis-spliced genes were genes involved in pre-mRNA and alternative mRNA splicing via the spliceosome. This suggests that ciliogenesis, cilium function, and pre-mRNA splicing are all regulated by alternative splicing in the retina, and this is defective in patients carrying *PRPF* mutations [51].

Other ciliopathy genes have been linked to RNA metabolism, such as *DDX59*, mutated in OFD [52-54]. DDX59 is a DEAD-box RNA helicase, of which there are more than 40 in humans [55]. DEAD-box RNA helicases can unwind short segments of double stranded RNA [56] and are involved in remodeling RNPs [57] for pre-mRNA splicing, RNA nuclear export, and ribosomal biogenesis [58]. Homozygous deleterious missense variants in the helicase ATP binding domain or helicase C terminus of DDX59 are associated with OFD [52]. These pathogenic variants in *DDX59* in OFD patients are associated with normal DDX59 cellular localisation, in a punctate distribution across the nucleus and cytoplasm, and normal ciliogenesis but abnormal ciliary signaling [52]. In *DDX59*$^{-/-}$ patient fibroblasts, cilia grew normally but did not respond appropriately to Hedgehog pathway stimulation, measured by Gli1 expression levels after treatment with Smoothened agonist (SAG) [52]. Variants causing loss of the stop codon in *DDX59* are also associated with OFD, with additional features [53]. Null variants in *DDX59* are associated with a more severe phenotype of OFD with complex neurological involvement, including structural brain anomalies, seizures and global developmental delay [54]. The structural brain abnormalities are similar to those seen in CADASIL syndrome, caused by mutations in the *NOTCH3* gene, leading to speculation that DDX59, through regulation of the primary cilium, plays a role in notch signalling regulation [54]. Loss-of-function *Drosophila* mutants of the homologue of *DDX59* display severe neurodevelopmental defects including gross disorganisation of the peripheral nervous system during development, loss or incomplete ventral nerve cord and shortened lifespan [54]. The data on DDX59 would seem to suggest that this protein, with roles across multiple processes in RNA metabolism, is crucially important for normal embryonic development, in particular, formation of midline structures. The exact molecular mechanism of the role of DDX59 in ciliary function remains

unclear and requires further study but supports the hypothesis that RNA processing is of core importance to ciliary function.

Splicing in the pathogenesis of ciliopathies – splice variants in ciliopathy genes

Normal pre-mRNA splicing and alternative splicing confer healthy diversity and complexity to a range of cellular functions. Splicing is a tightly regulated process, controlled by a range of protein and RNA splicing regulators [59]. Recognition of splice sites depends on conserved sequences around splice sites, and in introns and exons [60]. At each splice site a consensus sequence at the DNA level defines the intron/exon boundary, and the branch site in the intron defines the location of binding of the 5' splice site to the intronic sequence to form the lariat before intron removal [61, 62]. As well as the consensus splice site sequences and the branch point, sequence-specific exonic splice enhancers (ESEs) and intronic splice enhancers (ISEs)[63] promote splicing, and sequence-specific exonic splice silencers (ESSs)[64] and intronic splice silencers (ISSs) [65] inhibit splicing. In spite of tight regulation of the splicing process, specific inherited genetic variants which disrupt the consensus splice site, branchpoint, ESE, ISE, ESS or ISS can lead to aberrant splicing. Such aberrant splicing may constitute the abnormal use of 3' or 5' splice sites, aberrant inclusion of introns or exon skipping and contributes to abnormal cell functions and a range of human diseases [66, 67].

At least 188 different genes can cause a syndromic or non-syndromic ciliopathy phenotype (**Table 2**). Pathogenic splice variants have been reported in more than half (103/188 i.e. 54.79%) of the known ciliopathy genes (**Table 2**). Pathogenic splice variants are particularly common in certain genes. For example, 25% of all published disease-causing variants in *MKS1* affect splicing, and 44.4% of published disease-causing variants in *RSPH1* affect splicing (**Table 2**) [68, 69]. In the case of these two genes, splicing variants are more commonly associated with disease because they cause a loss-of-function, and such loss-of-function variants are sufficient to cause disease whilst missense variants tend to be tolerated and do not cause disease, or cause a less severe disease. Null variants in *MKS1* are a common cause of MKS [70], whilst null variants in *RSPH1* cause a mild form of PCD [71]. Missense variants in *MKS1* have been reported as a very rare cause of BBS [72], but missense variants in *RSPH1* have not been reported as a cause of disease. Similarly to *RSPH1*, loss-of-function variants (but not missense variants) in *CCDC114* cause PCD, and pathogenic loss-of-function splice variants in this gene are common [73]. There are several other ciliopathy disease genes where splice variants are slightly more common because only loss-of-function variants cause disease and missense variants have not been reported as causing disease. These tend to be associated with the severe ciliopathies, and include; *CEP104* and *TMEM237* which cause JBTS [74, 75]; CSPP1 which causes MKS and JBTS with occasional features of SRPS [76-78]; *CCDC39*, *CCDC40*, *HYDIN* and *DNAI2* which cause PCD [79-81]. Occasionally, pathogenic splice variants cause less severe disease than nonsense or frameshift mutations, because the transcript produced by the splice variant remains in-frame. For example, study of the spectrum of mutations in *OFD1* shows that missense and particular splice site mutation (c.2260 + 2 T > G at splice donor site of intron 16, leading to 513bp in-frame deletion) are associated with OFD in males [82], whereas this X-linked condition is usually associated with male lethality in the case of null mutations, including splicing mutations causing frameshifts [83]. A splice site mutation in *TCTN3* which causes in-frame skipping of exon 7 causes JBTS, whereas nonsense or frameshift mutations cause MKS [84]. Pathogenic splice variants are also common in *C8orf37*, which causes BBS, CORD and RP. Particular splice mutations in this gene, such as c.156-2A>G are associated with polydactyly along with CORD [85-89]. It has been postulated that splicing variants impact the transcriptional profile of disease genes

in a more highly variable manner than other types of genetic variant, and due to tissue-specific transcript expression, this could partly account for the broad phenotypic variability observed in ciliopathies, even within families, such as MKS associated with *MKS1* variants [68]. This could help to explain the very broad phenotypic spectrum of conditions from fetal encephalocele to Joubert-related syndrome to OFD, associated with variants in *C2CD3*, which are frequently splice-altering variants which cause frameshifts in the transcript(s) [90]. Around 20% of cases of Joubert syndrome are caused by variants in *AHI1*, of which around 15% are splice site mutations [91]. *BBS1*, the most common cause of BBS (in around 40% of cases) can be associated with mutations at splice donor site in exon 4 (432+1G>A) [92]. The mechanism underlying the higher frequency of splicing variants in certain genes than others remains unclear. Individual splicing changes are also common in certain genes, such as *CEP290*, with the same variant observed repeatedly in many individuals with disease. A deep intronic *CEP290* variant (c.2991+1655A>G), which creates a strong splice-donor site and inserts a cryptic exon in the *CEP290* messenger RNA, is detected in 21% of all LCA patients [93]. 60 -90% of LCA patients with *CEP290* mutations have at least one c.2991+1655A>G allele [94-96]. Similarly, a deep intronic variant in *USH2A* (c.7595-2144A>G) which introduces a novel splice donor site in intron 40, leading to insertion of a pseduoexon, PE40, [97] is the second most common cause of USH type 2A, with a frequency of 4% [98]. Three other deep intronic variants in *USH2A* have been reported as causes of USH type 2A, but these are less common [99]. Variants in *USH2A* are one of the most common causes of ARRP, but whilst splice-site variants in *USH2A* have been reported as a cause of RP, to date no deep intronic variants in *USH2A* have been reported in autosomal recessive RP (AR-RP) patients [100, 101]. A founder mutation, a 29-bp deletion in intron 15 of *MKS1*, is a common cause of MKS in European (and especially the Finnish) populations (the so-called 'Finn-major mutation') [70]. This particular splice mutation is associated with a form of MKS involving skeletal defects, which are rarer in other forms of MKS [102]. The most common genetic cause of MKS, *TMEM67*, has two splice founder mutations in the Pakistani population, (c.1546 + 1 G > A and c.870-2A > G)[103]. A +3 splice donor site mutation at exon 11 of *BBS1*, leading to use of an alternative cryptic donor site within the exon, is a common cause of BBS in the Faroe Islands due to a founder effect [104]. This variant is associated with severe, early onset retinal dystrophy, earlier than that seen in patients with different *BBS1* mutations [104]. In type 2 BBS, a c.472-2A>G splice acceptor site mutation in *BBS2* is a common disease variant in the Hutterite population [105]. The c.742G>A variant, abolishing the consensus splice donor site of exon 7, in *CCDC114* is a PCD founder mutation in the Dutch Volendam population [73] and an intron 2 c.921+3_6delAAGT splice donor mutation in *RSPH4A* is a common cause of PCD in individuals of Hispanic descent due to a founder effect [106]. A 3bp insertion at the exon 1/intron 1 splice donor site in *DNAI1* is a common variant in the Caucasian population, accounting for up to 55% of *DNAI1*-associated cases of PCD in white Europeans, with up to 82% of PCD patients having at least one of these alleles [107]. As *DNAI1* mutations account for up to 9% of PCD cases, this particular variant is significant in terms of its contribution to disease in this population [107]. There have also been reports of common exonic variants affecting splicing. A common Jewish founder mutation in *MAK*, caused by an Alu insertion in exon 9, leads to exon skipping of exons 9 and 12 from the final processed transcript. Exon 12 is a retina-specific exon which is important for specific protein function in the retina, and loss of this exon in patients carrying this Alu insertion leads to RP [108]. A non-synonymous exonic variant in *NPHP3* has also been shown to cause ciliopathy NPHP through aberrant splicing. The synonymous variant c.2154C>T; p.Phe718=, 18 base pairs from the exon–intron boundary within exon 15 of *NPHP3* causes skipping of exon 15 [109].

Genetic diagnosis of ciliopathies is largely achieved using gene panel or targeted exome sequencing. In less genetically diverse cohorts, diagnostic yields of up to 85% can be achieved [110]. However, typical diagnostic yields of 42.4% to 67.6% are achieved for motile ciliopathy patients [111, 112] and 55 to 62% for non-motile ciliopathies [113, 114]. It is likely that underestimation of the contribution of splice defects to the pathogenesis of ciliopathies contributes to limited diagnostic yields. Early estimates suggested that around 15% of pathogenic variants affect splicing [115]. However, intronic variants affecting splicing are often overlooked due to perceived technical difficulties in proving the pathogenicity of these variants. Indeed, when we reviewed pathogenic splice variants in ciliopathy genes reported in the literature (as collated in HGMD), [116] we found that on average around 17.5% of variants affect splicing. However, when we compared this to variants in clinical variant interpretation database ClinVar [48], only 6% of pathogenic variants affected splicing (**Table 2**). Many more variants affecting splicing were assigned 'uncertain significance' status. It seems clear that there remains uncertainty when assigning pathogenic status to splice variants, and this remains a barrier to increasing diagnostic yields in genetic testing. At this point in time, few diagnostic clinical genetics laboratories in the UK perform any splicing analysis at the level of RNA, and there are no clear guidelines on how to interpret genomic variants potentially affecting splicing.

In addition to lack of confidence in classifying intronic variants as altering splicing, it is likely that many exonic variants affecting splicing are misclassified as missense or non-pathogenic synonymous changes. An estimated 22-25% of exonic variants classified as missense or nonsense actually affect splicing [117, 118]. For example, compound heterozygous variants in *LZTFL1* (BBS17) predicted to introduce missense changes were actually found to produce truncated protein (35kDa instead of 30kDa), suggesting that these variants actually impact on splicing [119]. A predicted missense variant at the end of exon 9 of *CSPP1*, a gene which is associated with a range of severe ciliopathies, was found to abolish normal splicing, leading to inclusion of an additional 10bp of sequence, shifting the reading frame and introducing a premature stop codon [78]. A predicted missense variant at the end of exon 4 of *TMEM231* was also shown to be affecting splicing and causing disease in MKS [120]. A single base pair substitution at the start of exon 16 in *DNAI1*, predicted to cause a missense amino acid change, was also shown to affect splicing, leading to skipping of exons 15 and 16 in patients with PCD [107].

It remains challenging to accurately predict the pathogenicity of variants potentially affecting splicing. The best optimisation of *in silico* splice prediction tools to date (using a combination of MaxEntScan with a 15% cut-off value and the PWM model with a 5% cut-off value in a study of *BRCA1* and *BRCA2* variants) achieved a sensitivity of 96% and specificity of 83% [121]. In practice, the accuracy of *in silico* splice prediction tools is often significantly lower. The contribution of coding sequence variants affecting splicing (such as synonymous mutations affecting exonic splicing enhancers) and deep intronic changes affecting splicing are particularly likely to be underestimated as they are often filtered out in genetic analysis pipelines which focus on non-synonymous coding and splice site variants. Furthermore, exome sequencing and gene panel sequencing often do not provide sequencing coverage of intronic variants further than 10-20bp from the intron/exon junction. As whole genome sequencing is more routinely used for disease diagnostics, thanks in part to the UK's 100,000 Genomes Project, more deep intronic variants are being revealed as causes of disease. For example, four new deep intronic variants in *USH2A* have been described in recent years [122, 123].

Diagnosing ciliopathies using RNA sequence analysis

Whilst *in silico* tools can be used to predict the effect of genomic DNA sequence variants on splicing, the most reliable method for identifying splicing changes is through direct RNA sequence analysis. Traditionally this has involved targeted RT-PCR of regions identified as potentially pathogenic from genomic DNA analysis, using RNA from relevant patient tissues or using minigene assays. There are many examples where RT-PCR of patient RNA has been employed to diagnose ciliopathies across the phenotypic spectrum including, but not limited to ; a deep intronic mutation in *OFD1* as a cause of XL-RP [124]; an intron 3/exon 4 splice acceptor site mutation in *BBS2* as a cause of BBS [105]; c.505+2T>C in *B9D1* as a cause of MKS [125]; two different splice-altering variants in *CEP78* as a cause of USH [126]; three different splice-altering mutations in *DNAI1* as a cause of PCD and; a splice acceptor site mutation in *TCTN3* as a cause of JBTS [84]. Minigene assays have been used to confirm splicing defects as causes of disease in various ciliopathies, including; various splice mutations in *BBS2*, *BBS3*, *BBS4* and *ALMS1* causing BBS [127] and; c.3112-5T>A in *IFT127* in patients with isolated RP [128]. However, whilst minigene assays give some insight into the effect of mutations on splicing, they may not be truly representative of the exact splicing defect *in vivo*. In the case of this variant in *IFT172*, the minigene assay showed that this genetic variant led to intron 28 retention, whereas RNA extracted from patient lymphoblastoid cell lines showed differential usage of the 3' splice site rather than complete intron retention [128]. Massively parallel whole transcriptome RNA sequencing (RNAseq) is a powerful technology which has the potential to revolutionise splice analysis, either in combination with genomic DNA sequence analysis, or as a standalone method. It has the potential to enhance diagnostic yields through direct detection of splicing aberrations caused by pathogenic variants, without the requisite prior knowledge for targeting RT-PCR. As a relatively new technology, there is little consensus on usage, interpretation, reliability or best practices for transcript-level analysis in RNAseq datasets, particularly for the purposes of novel transcript or novel splicing event identification, which is critical for the successful application of RNAseq to disease diagnostics.

One of the major fundamental challenges of analysing RNAseq data is accurately assembling long transcripts from short reads of sequence. Furthermore, estimation of isoform abundance from short sequence reads is statistically challenging, as each read samples only a small part of the transcript, and alternative transcripts often have substantial overlap. Mapping of reads to exons can also be challenging when there is differential 3' and 5' splice site usage. In the case of detecting novel splice events, particularly rare, disease specific events, or those which may be undergoing nonsense mediated decay (NMD), managing the balance between sensitivity and specificity is vital, to avoid overlooking disease causing events or having true events be swamped by false positives. This presents a major challenge for diagnosis of ciliopathies associated with genes with large transcript diversity, such as *IFT122*, associated with a form of SRPS. *IFT122* has at least four differentially spliced transcripts. One reported exon 6/intron 6 splice donor site mutation c.502+5G>A in *IFT122*, which causes exon 6 skipping, has been shown to only affect transcript isoform 3 (which includes exon 6), but not transcript isoform 4 (which does not include exon 6) [129]. Difficulty in differentiating between different transcripts using RNAseq could compromise the ability to detect pathogenic mutations such as this. In a separate case, amplification of cDNA from RNA from a parent carrying a splice-site variant in *TMEM231* was shown to display complete loss of heterozygosity of the disease allele, due to complete NMD of the aberrant transcript [120]. This highlights the potential challenges of capturing and identifying disease-associated RNA transcripts for the purposes of ciliopathy diagnostics. A further challenge stems from the tissue-specific nature of the expression and splicing of many ciliopathy

genes, in tissues which are not easily accessible and/or frequently biopsied during the course of care (e.g. blood, fibroblasts, muscle tissue). For example, only two of the recognised transcript isoforms of *IFT122* are expressed in blood, and any splice variant affecting other transcripts would not be detected by RNAseq performed on whole blood [129]. To address this issue, studies are increasingly expanding to extract RNA from more diverse patient materials, including skin biopsies/fibroblasts and urine-derived renal epithelial cells (URECs), which provides ciliated patient cells in a non-invasive manner [130]. This has been successfully applied in identifying variants causing exon 15 skipping in *NPHP3* in URECs. RT-PCR revealed wild-type (WT) mRNA from URECs harboured only transcripts containing exon 15, while heterozygous variant-carrying URECs from the patient's father showed an additional transcript with this exon being spliced out. In mRNA from blood, exon 15 is spliced out in healthy controls, which would likely have masked the variant's effect had this been the tissue of choice [109]. However, the success of this approach depends on robust transcriptional data from these tissues from healthy controls, and this data is lacking for many disease-relavant tissues. For example, in the study and diagnosis of PCD, multiciliated airway cells are obtained from patients through nasal brushings, which can be sampled fresh or grown at air-liquid interface (ALI) to generate a larger bank of patient material [131], although ALI cultures show different phenotypes from fresh samples [132]. These samples are currently used for diagnostic imaging, but work is ongoing by our groups and others to characterise the transcriptome of control samples in order to permit RNA sequencing of patient samples to enhance genetic diagnosis of PCD. The issue of tissue-specific transcript expression is a particular challenge in diagnosis of retinal ciliopathies. As previously discussed, the retina, and photoreceptor cells in particular, exhibit highly tissue-specific transcriptional profiles, with many ciliopathy genes producing exclusively photoreceptor-specific isoforms which are specifically affected by disease-causing splice mutations which cannot be confirmed by RNA analysis from other tissues. Advances in retinal organoid culture techniques from induced pluripotent stem cells derived from patient fibroblasts have provided one solution to this problem, but this is an extremely laborious and time-consuming process, taking several months for retinal organoid cultures to develop mature photoreceptors [133]. Culture techniques are being accelerated [134], refined towards a standardised approach, and the transcriptional profile of these organoids being defined [135], including using single cell RNAseq to understand the transcriptional profile of different cell types of these retinal organoids [136]. As a result, retinal organoids are providing useful models for studying mechanisms of retinal disease [51] and effectiveness of novel molecular therapies [137] and diagnostics; they have recently been used to characterise the effect of a novel splice acceptor site variant in *USH2A* as a cause of RP [138].

Dozens of computational methods for RNAseq analysis have been developed, each with their own individual strengths and limitations. A comprehensive discussion of all available computational approaches is beyond the scope of this paper (see e.g. [139-141]), and we will focus only on a basic discussion of those that have been applied to splicing analysis for rare disease diagnostics to date. Four recent papers have been published exploring the application of RNAseq for diagnostic purposes in various Mendelian diseases. Cummings *et al*. and Gonorazky *et al*. both focussed on neuromuscular disorders, obtaining diagnostic yields of 35-36% from RNAseq on biopsied muscle tissue [142, 143]. The methodology utilised involved alignment using STAR's two-pass method [144], followed by filtering and prioritisation of splice junctions of potential diagnostic relevance using code developed by Cummings *et al.*, along with investigating allelic imbalance in expression. Kremer *et al*.'s focus was mitochondrial disorders, with RNAseq performed on patient derived fibroblasts [145]. Again, STAR

was the alignment method of choice, coupled with LeafCutter[146] for detection and prioritisation of aberrant splicing events, and investigation of aberrant and monoallelic expression was done, achieving a diagnostic result in 10% of patients. Finally, Frésard *et al*. used RNAseq on blood mRNA in the diagnosis of a cohort of patients with a diverse range of rare diseases spanning 11 categories, including neurology, hematology and ophthalmology [147]. Using a combination of techniques including detection of expression and splicing outliers, a diagnostic rate of 8.5% was obtained. Candidate diagnoses were also identified for several other patients, including those with neurological phenotypes, for whom blood would not be an obvious tissue of interest, demonstrating the potential for wider applicability, albeit with a lower diagnostic yield.

Common themes in the analysis strategies in the above papers include the use of STAR for alignment, and the leveraging of publically available RNAseq data (e.g. GTEx https://gtexportal.org/) as controls. Despite many different alignment tools being available, STAR appears to be emerging as a front-runner for RNAseq, likely due to its speed and ease of use, clear documentation and active support. STAR in two-pass mode utilises known splice junctions from a provided transcriptome, identifying potential novel junctions well supported by split reads (those spanning a splice junction) in the data on the first pass, then re-traverses the data to provide read counts for all junctions[144]. Utilising publically available RNAseq data, which most of the above papers do, is a powerful strategy for reducing noise and removing from analysis splice junctions that are present in non-diseased individuals and are thus unlikely to be the aberrant splicing events responsible for disease in the patient in question. Careful matching of tissue type and study design, and reprocessing of control data alongside cases can help ensure maximum consistency between different datasets.

A further issue to resolve to optimise the use of transcriptomics for rare disease diagnostics is the depth of sequencing required to achieve robust detection of rare splice forms. At low sequencing depth, RNA sequencing experiments likely fail to detect the majority of low-abundance transcripts, which only become apparent through deep RNA sequencing. Typical differential gene expression analysis studies sequence to a depth of 20 million reads per sample. In the retina, only approximately 50% of exons will be covered with sequencing at this depth [25]. For splicing analysis, read depths of 50-100 million reads per sample have typically been employed [142, 143], as greater depths are required to detect rare, patient specific splicing events, and those that may be undergoing NMD.

As more research is undertaken using RNAseq diagnostically, larger and more diverse datasets will facilitate the development of optimal methods both in terms of study design and analytical practice. Responsible sharing of data will be crucial in maximising both broad scientific and diagnostic benefit. In the UK, the NIHR have recently funded a program of research into clincial diagnostic uplift in a range of genetic disorders, including PCD, where best practice guidelines for the effective utilisation of RNAseq in a diagnostic setting will be explored.

Treating ciliopathies by targeting splicing

Ciliopathies have long been considered untreatable and incurable conditions. Recent advances in gene therapy have challenged this view. Early efforts have focussed on gene delivery by viral vectors for treatment of the most common causes of recessive retinal ciliopathies. This has led to two

independent clinical trials for treatment of the RP phenotype in USH patients carrying *MYO7A* mutations (https://clinicaltrials.gov/ct2/show/NCT02065011; https://clinicaltrials.gov/ct2/show/NCT01505062), and two independent Phase1/2 clinical trials for treatment of XL-RP associated with mutations in *RPGR* (https://clinicaltrials.gov/ct2/show/NCT03316560; https://clinicaltrials.gov/ct2/show/NCT03252847?cond=Retinitis+Pigmentosa&rank=38). Excitingly, a third *RPGR* gene augmentation trial is now recruiting for Phase2/3 (https://clinicaltrials.gov/ct2/show/NCT03116113). *RPGR* mutations are responsible for 70-90% of XL-RP cases, and 10-20% of all RP cases [40]. Mutations in *MYO7A* account for around 20% of USH cases [148]. However, some of the most common genetic causes of ciliopathies are very large genes which cannot be delivered by viral vectors. Adeno-associated virus serotype 2 (AAV2), the commonly used viral vector, is limited to delivery of around 3.5kb of genetic material, including promoter and polyadenylation sequence [149]. *USH2A*, which accounts for 10 to 15% of cases of AR-RP and 50% of USH cases, has a 12kb coding sequence. The most common genetic cause of ciliopathy, *PKD1*, which accounts for 85% of cases of ADPKD, has a coding region of almost 13kb.

As a result, alternative approaches are now under investigation, many of which focus on altering splicing. Antisense oligonucleotides (ASOs) are one technology which can be used to modulate splicing. ASOs are short, single-stranded synthetic oligodeoxynucleotides which are designed to target specific regions of mRNA to modulate splicing to correct genetic defects causing disease. They can be used to promote retention of exons that are otherwise skipped in affected patients, and skip pseudoexons which are otherwise retained in affected patients [150]. ASOs can also be used to induce skipping of exons carrying premature stop codons or insertions/deletions causing a frameshift, to restore the reading frame and restore protein production. Indeed, this approach has led to the first FDA-approved ASO for treatment of Duchenne Muscular Dystrophy (DMD), eteplirsen [151] and the first FDA-approved ASO for treatment of Spinal Muscular Atrophy (SMA), nusinersen [152].Eteplirsen is an ASO designed to induce exon 51 skipping of *DMD* pre-mRNA, which encodes dystrophin, which is mutated in patients with DMD [153]. Eteplirsen causes exon 51 skipping which restores the reading frame of *DMD* in DMD patients with out-of-frame deletions in the *DMD* gene, restoring dystrophin protein production [154, 155]. The protein produced is truncated, but this is sufficient to reduce the severity of patient phenotype, slowing respiratory decline [156] and extending the number of years a patient remains ambulatory compared to untreated identical twin patients [157]. Nusinersen is an ASO designed to promote exon 7 inclusion in mature *SMN2* mRNA through blocking the binding site of hnRNP in intron 7 of *SMN2* pre-mRNA[158, 159]. This hnRNP binding site acts as a splicing silencer in patients with SMA, resulting in exclusion of exon 7 and truncated protein production. Nusinersen restores full-length protein production and improves motor function and probability of survival in treated early-onset SMA patients compared to sham control [152, 160]. It is also successful in improving motor function in later-onset SMA patients [161].

ASO technology has improved in recent years to reduce off-target effects and toxicity, improve nuclease resistance and target-binding affinity [162]. This has been achieved through modification of the ASO backbone such as 2' ribose modifications e.g. 2'-*O*-methylation (OMe), 2'-O-methoxy-ethylation (MOE), and locked nucleic acid (LNA), and P backbone modifications such as phosphorothioate and morpholino [163, 164] (**Figure 3**). Conjugation of the backbone to a carrier or ligand, such as lipids, peptides, carbohydrates or antibodies, has helped to improve bioavailablity and

delivery across membranes, and tissue-specific delivery [165, 166]. These improvements are making successful clinical applications more likely. For example, FDA-approved eteplirsen is a phosphorodiamidate morpholino oligonucleotide [151, 153, 154] and nusinersen is a 2′-O-(2-methoxyethyl) modified ASO.

The ASO treatment approach is particularly useful in conditions associated with a common mutation which affects splicing. There are several common founder mutations in *USH2A*, including deep intronic c.7595-2144A>G and which introduces a novel splice donor site in intron 40, leading to inclusion of a novel exon (PE40) in the mature transcript [123]. A recent study showed successful specific induction of exon skipping of this PE40 in patient fibroblasts and in a minigene assay [98]. A first-in-man study of the safety and tolerability of RNA ASO therapy for RP and USH patients with mutations in exon 13 of *USH2A* is currently underway, coordinated by the University of Michigan Kellogg Eye Centre and Retina Foundation of the Southwest, Dallas (https://clinicaltrials.gov/ct2/show/NCT03780257). This study is testing QR-421a, which targets exon 13 to induce exon 13 skipping in patients with nonsense or frame-shift mutations in exon 13 of *USH2A*, including the common c.2299delG and the c.2276G>T mutations (https://databases.lovd.nl/shared/variants/USH2A/). This aims to restore the reading from of *USH2A* mRNA and restore functional usherin protein production in the retina of patients to prevent retinal degeneration and vision loss. Another common mutation which affects splicing in ciliopathy patients, is the deep intronic variant c.2991+1655A>G (sometimes called IVS26) in *CEP290* which accounts for 20-25% of cases of LCA [93]. The change introduces a novel splice donor site and insertion of a novel cryptic exon, exon X, in the transcript, creating a premature truncation codon p.(Cys998*), probably subjecting the transcript to NMD (**Figure 5a**). A proof-of-principle study in 2012 showed the efficacy of an ASO approach in correcting *CEP290* splicing in human patient fibroblasts by blocking exon X to exclude it from the final transcript and restore WT transcript production [167]. A recent study has shown success of clinical drug candidate ASO QR-110, a single-stranded, fully phosphorothioated, and 2′ *O*-methyl-modified RNA oligonucleotide (**Figure 3**), in rescuing the *CEP290* splicing defect by blocking exon X splicing to restore WT CEP20 mRNA production in pre-clinical models including human patient-derived retinal organoids [137]. QR-110 was the most successful of 29 ASOs designed to target the region of exon X using an ‘oligo-walk‘ approach [137]. This led to a phase 1/2 clinical trial of intravitreal injection of QR-110, (https://clinicaltrials.gov/ct2/show/NCT03140969) which showed safety and tolerability, and an exceptional improvement in vision in one patient [168]. This is now entering phase 2/3 clinical trials (https://clinicaltrials.gov/ct2/show/NCT03913143).

Whilst initial efforts have focussed on treating the retina, as it is a relatively small area to treat and relatively minor improvements in cell functionality can lead to significant clinical outcomes for patients, work is now expanding into exploring systemic treatment of syndromic ciliopathies. In the first study of its kind worldwide, Prof John Sayer and colleagues showed effectiveness of ASO-induced splicing of a mutated exon (41, G1890*) of *CEP290* restoring CEP290 protein expression in kidney cells of JBTS patients. In treated cells, protein localises correctly to cilia and restores normal cilium length, whereas patient cilia overgrow in length. Systemic treatment in a Cep290 mouse model led to reduced cystic burden in the kidney [169].

Gene editing approaches are also being trialled as methods for correcting pathogenic splice variants in ciliopathy patients. This has the advantage of stably and permanently correcting the genome of patients. A recent study reported successful editing of the common intronic mutation in *CEP290* in

humanised CEP290 mutant mice. Adeno associated virus 5 (AAV5) was used to deliver two guide RNAs and *Staphyllococcus aureus* Cas9 to delete or invert the region of exon 15 containing the common intronic mutation in *CEP290* [170]. Sub-retinal injection of this therapeutic, named EDIT-101, into humanised CEP290 mutant mice resulted in efficient and specific CEP290 gene editing, but it is unclear whether this led to clinically significant improvements in visual function. EDIT-101 is now entering phase 1/2 clinical trials (https://clinicaltrials.gov/ct2/show/NCT03872479).

Spliceosome-mediated RNA *Trans*-splicing (SMaRT) [171, 172] is an alternative approach to rescuing splicing defects in ciliopathy patients. This is a modified gene delivery approach which involves delivering a partial coding DNA sequence (a 'pre-mRNA trans-splicing molecule' or PTM) designed to *trans*-splice to endogenous pre-mRNA. A single PTM is delivered by a viral vector to target endogenous pre-mRNA transcripts to yield a hybrid exogenous-endogenous mRNA which either excludes a pathogenic cryptic exon, an exon carrying a stop or frameshift mutation, or includes a skipped exon. This represents a safer approach than gene delivery which can result in toxic levels of overexpressed protein, or gene editing with dangers of off-target nuclease activity. A further advantage is that this approach can correct a larger range of mutations with one single therapeutic, which may be particularly helpful for patients with compound heterozygous mutations. A recent study has shown the effectiveness of this approach in correcting the mis-splicing caused by *CEP290* c.2991+1655A>G *in vitro* and *in vivo* in a mouse minigene model of *CEP290* intron 26-27 (**Figure 4**) [173]. In this study, the PTM includes a CMV promotor, a codon optimised partial coding sequence (PCDS) of *CEP290* cDNA from position 1 (A of ATG) to 2991, a 5' splice site and a sequence complementary to intron 26-27 of CEP290, upstream of c.2991+1655A>G, and a polyA tail to aid entry into the spliceosome. When transfected into cells, this minigene is expressed to produce an RNA molecule consisting of the PCDS and a binding domain which complementarily binds to endogenous *CEP290* pre-mRNA upstream of the c.2991+1655A>G mutation (**Figure 4b**). The RNA product from the minigene and the endogenous *CEP290* pre-mRNA then undergo *trans*-splicing in the spliceosome to produce a hybrid *CEP290* mRNA which includes minigene-derived PCDS sequence from exons 1-26 and endogenous sequence from exon 27-54, with exon X excluded (bottom schematic in **Figure 4c**) which encodes full-length wild-type CEP290 protein. Thus, trans-splicing bypasses the effect of the c.2991+1655A>G mutation and restores full-length wild-type CEP290 protein production [173].

Delivery of modified U1 snRNA to cells affected by a pathogenic splicing change can also be used to correct splice defects. U1 snRNA binds the splice donor site to initiate exon recognition during splicing, and delivery of a U1 snRNA optimised with increased binding affinity to a mutant splice donor site can repair splicing. Aberrant splicing of exon 5 of *BBS1*, associated with a splice donor site mutation, was partially corrected in patient fibroblasts by lentiviral delivery of a sequence-modified synthetic U1 snRNA in a dose-dependent manner [174]. Similarly, this therapeutic U1 snRNA approach has been used to correct mis-splicing of *RPGR* in patient fibroblasts with point mutation in exon 10 [174, 175]. This is a promising strategy for treatment of patients with splice mutations in *BBS1* or RPGR, the most common genetic cause of BBS and XL- RP respectively. However, these have not progressed towards gene therapy as of yet.

Conclusion and future perspective

Splicing contributes significantly to the pathogenesis, diagnosis and future treatment strategies of ciliopathies. Pathogenic splice variants have been reported in more than half of the known ciliopathy

genes (**Table 1**), and there is probably an underestimate of the contribution of splicing defects to ciliopathies due to difficulty in deciding whether a 'splice variant' is pathogenic. When we reviewed pathogenic splice variants in ciliopathy genes reported in the literature (as collated in HGMD, Stenson et al., 2003) we found that on average around 17.5% of variants affect splicing. However, when we compared this to variants in clinical variant interpretation database ClinVar (Landrum et al., 2014), only 6% of pathogenic variants affected splicing (**Table 1**). Many more variants affecting splicing were assigned 'uncertain significance' status. It seems clear that there remains uncertainty when assigning pathogenic status to splice variants, and this remains a barrier to increasing diagnostic yields in genetic testing. At this point in time, few diagnostic clinical genetics laboratories in the UK perform any splicing analysis at the level of RNA, with no commissioned clinical service and no clear guidelines on how to interpret genomic variants potentially affecting splicing. At this critical time, and with the advent of the integration of next generation sequencing into clinical practice, it is important to incorporate clinical RNA analyses to maximise diagnostic uplift for patient benefit. Indeed for some disorders this may mean RNA first and DNA second. For ciliopathies in particular we can achieve this by building a robust body of knowledge of the control of the transcriptome and how this changes in disease - a form of 'biomarker'. Development of both the transcriptome methods and pipelines of analyses as well as clear guidelines for interpretation will be essential. Finally, as we improve our understanding of how sequence changes affecting splicing cause disease, we will better understand the complex mechanism of splicing, facilitating development of innovative methods for manipulation of the splicing process, revealing future therapeutic targets.

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Figure and Table legends

Figure 1: Schematic representation of primary cilium, motile cilia and photoreceptor cilium
(**a**) Schematic figure of a typical epithelial cell with single apical non-motile primary cilium. Cilium membrane in orange, microtubule doublet axoneme and triplet basal body in red. (**b**) Cross-section of axoneme of non-motile primary cilium showing radial arrangement of microtubule doublets in red. (**c**) Schematic figure of a rod photoreceptor cell with highly modified cilium. Cilium membrane in orange, microtubule doublet axoneme and triplet basal body in red, membrane stacks with rhodopsin molecules as purple dots. (**d**) Cross-section of axoneme of photoreceptor cilium showing radial arrangement of microtubule doublets in red. (**e**) Schematic figure of a typical epithelial cell with multiple apical motile cilia. Cilium membrane in orange, microtubule doublet axoneme in red. (**f**) Cross-section of axoneme of non-motile primary cilium showing radial arrangement of microtubule doublets plus central pair of microtubules in red, nexin links in navy, radial spokes in purple, and inner and outer dynein arms in green.

Figure 2: Typical clinical features of ciliopathies
(a-c) Brain MRI findings for an individual with Joubert syndrome, showing the molar tooth sign with moderate vermis hypoplasia, elevated and thickened superior cerebellar peduncles (arrowhead), and superior cerebellar dysplasia (arrow) indicated. Reproduced from Wheway et al., 2015. **(d, e)** Clinical features of individuals with a short-rib thoracic dystrophy, including narrow and deformed thorax. Reproduced from Wheway et al., 2015. (**f**) Clinical pictures of a male Alstrom syndrome patient at age 6 years 8 months, presenting with truncal obesity. Note characteristic face and prominent ears. Reproduced from Marshall et al., 2011 under the terms of the Creative Commons Attribution License CC BY. **(g)** Massive swelling of the abdomen of a foetus at gestation age 18+/40 with Meckel-Gruber

syndrome due to grossly enlarged, cystic kidneys. Reproduced from Hartill et al., 2017 under the terms of the Creative Commons Attribution License CC BY. **(h)** Cystic dysplasia of the kidneys comprising large, fluid-filled cysts, small cysts and cystic swelling of the proximal tubules and glomeruli, with absence of normal renal parenchyma in MKS. Reproduced from Hartill et al., 2017 under the terms of the Creative Commons Attribution License CC BY. (i, j) postaxial polydactyly on both hands of a patient with orofaciodigital syndrome. Reproduced with permission from Bonnard et al., 2018. (**k, l**) Examples of fundoscopy images revealing mild pigmentary depositions and some mottling in syndromic ciliopathy patients with retinal dystophy. Reproduced from Wheway et al., 2015. (**m**) coronal computed tomography (CT) scan of a 17-year-old PCD patient showing diffuse sinusitis with mucosal thickening and polyposis. **(n)** Endoscopic view showing nasal polyp in a PCD patient **(o)** Chest X-ray of a 6-year-old PCD individual with middle lobe atelectasis. Silhouetting of the right heart border is present. **(p)** Chest CT scan of a 6-year-old individual with *situs inversus totalis*. The left-sided middle lobe shows extensive bronchiectasis with volume loss (white arrowhead). In addition, consolidations and mucous impaction are present in the right upper lobe. Reproduced from Werner et al., 2015 under the terms of the Creative Commons Attribution License CC BY.

Figure 3. Oligonucleotide backbones for antisense oligonucleotide therapeutics

The figure shows basic chemical structure of an unmodified DNA or RNA molecule, and modified structures (with modification highlighted with pink circle) used for antisense oligonucleotide therapeutic treatment of ciliopathies.

Figure 5. Strategy to rescue aberrant *CEP290* splicing through spliceosome-mediated 5′ Pre-mRNA *trans*-Splicing

(**a**) Diagram of the most prevalent mutation in Leber congenital amaurosis type 10 c.2991+1655A>G, which introduces a new intronic canonical 5′ splice site (5′ SS). The novel splice site leads to inclusion of a cryptic exon, exon X. This cryptic exon encodes a premature stop codon (black octagon) leading to a truncated protein. (**b**) Schematic of an approach to utilize a 5′ pre-mRNA *trans*-splicing molecule (PTM) to rescue mutations in *CEP290* that are located 5′ to intron X-27. The PTM transcript consists of the partial coding DNA sequence (PCDS) encoding CEP290 exons 1-26, the novel 5' SS, a spacer and a 'putative binding domain', which is reverse complementary to the target sequence in intron X-27. (**c**) Three potential splicing outcomes with *CEP290* c.2991+1655A>G following introduction of a 5′ PTM: (1) joining of exon 26 to exon 27 from *cis*-splicing for the wild-type junction; (2) inclusion of exon X from *cis*-splicing (predominant mRNA species with c.2991+1655A>G present); (3) joining of the 5′ PCDS to exon 27 from *trans*-splicing. Both outcomes 1 and 3 would result in full-length CEP290 peptide (because the PCDS is designed such that it encodes exons 1-26). Reproduced with modification from Dooley et al., 2018 under the terms of the Creative Commons Attribution License CC-BY.

Table 1. Class, OMIM phenotype number, disease genes and hallmark clinical features of major ciliopathies

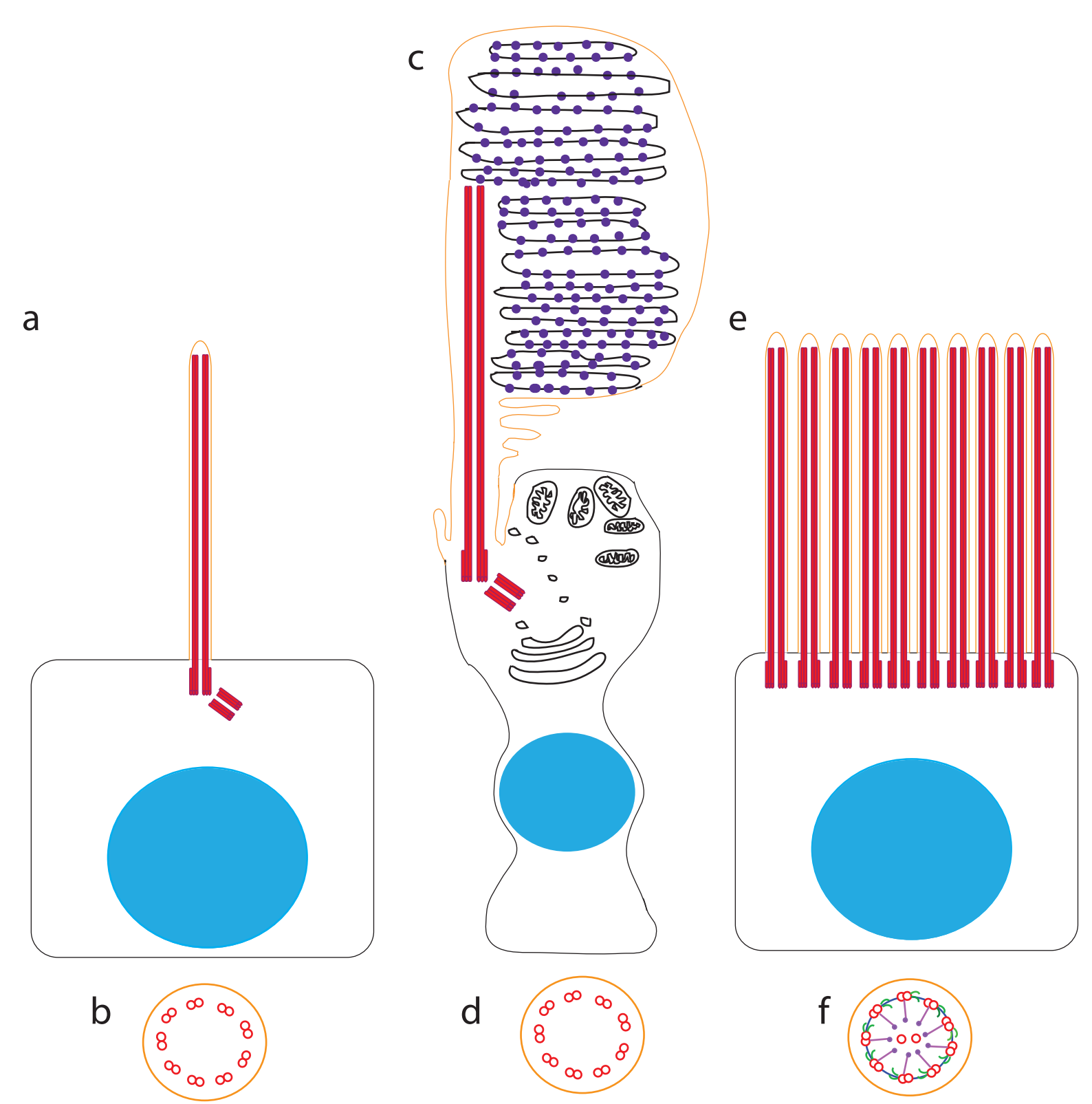
Major ciliopathies, grouped into non-motile and motile, subdivided into neurodevelopmental, skeletal, obesity, sensorineural, retinal and respiratory. Names, abbreviations and OMIM phenotypes are given for each, alongside a list on currently known disease genes associated with each condition, and hallmark clinical features.

Table 2. Detailed phenotypes and types of mutation reported in known ciliopathy genes

List of known ciliopathy genes, OMIM gene number, ciliopathy(s) associated with this gene, types of mutation reported, and percentage of reported mutations which affect splicing according to HGMD (Stenson et al., 2003) and ClinVar. Half of all ciliopathy genes (104/188) have at least one reported splicing variant associated with disease. Of the genes in which splicing variants have been reported in the literature these account for on average 17% of total reported variants. Clinical interpretation of splicing variants would seem to underestimate the pathogenicity of such variants.

Table 3. Ciliopathy abbreviations, full names and incidence rates per 100,000 in the general population

Abbreviation of ciliopathy, full name and estimated prevalence, from OMIM, Orphanet Rare Disease or published literature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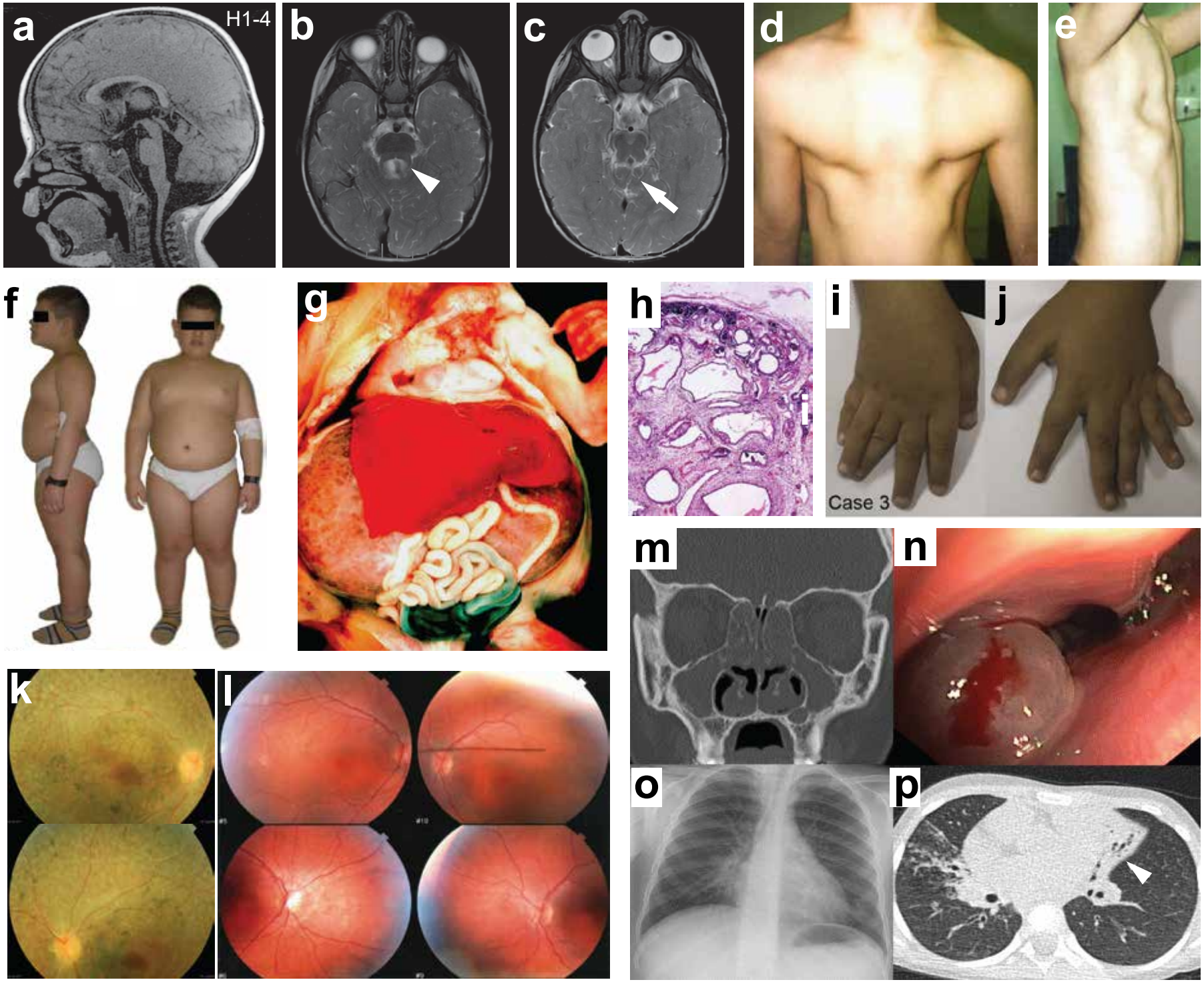
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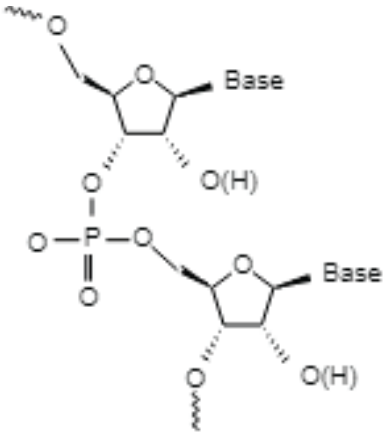

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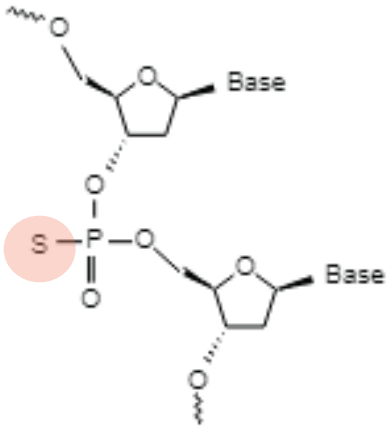

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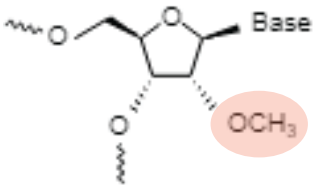

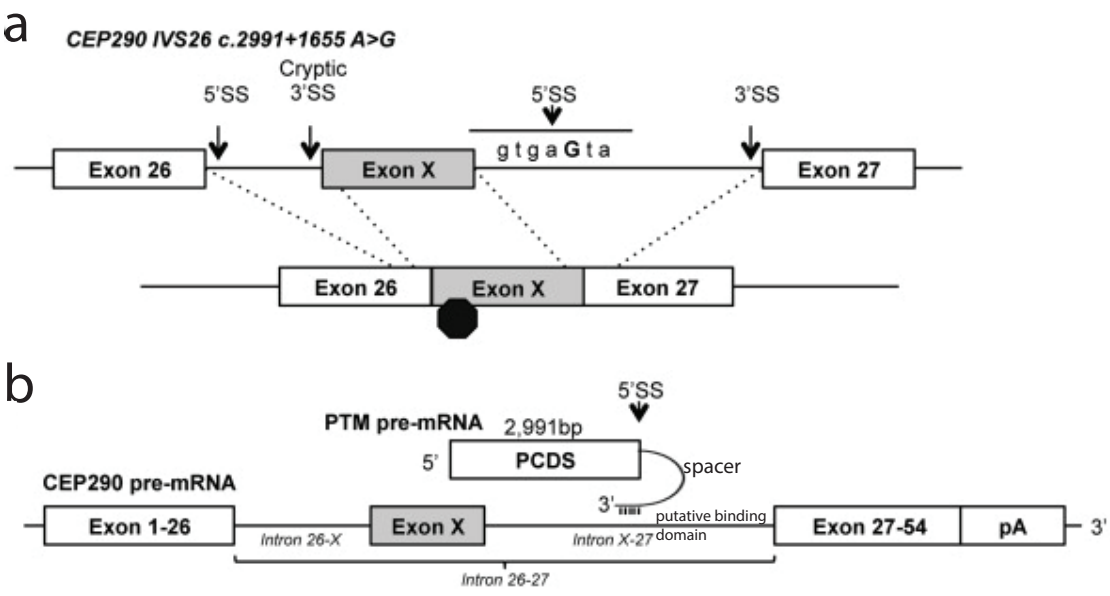
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Exon 26
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Exon 27
Exon 26
Exon X
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b
5'SS
PTM pre-mRNA
2,991bp
5'
PCDS
spacer
3'
putative binding domain
CEP290 pre-mRNA
Exon 1-26
Intron 26-X
Exon X
Intron X-27
Exon 27-54
pA
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Potential mRNA Outcomes of RNA Editing
Exon 1-26
Exon 27-54
AAAA
cis-wild-type
290.4 kDa CEP290
Exon 1-26
Exon X
Exon 27-54
AAAA
cis-mutant
PCDS
Exon 27-54
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trans-hybrid
290.4 kDa CEP290

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Based on the above disclosures, this form will automatically generate a disclosure statement, which will appear in the box below.

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ICMJE Form for Disclosure of Potential Conflicts of Interest

Instructions

The purpose [illegible] influence how they receive and understand your work. The form is designed to be completed electronically and stored electronically. It contains programming that allows appropriate data display. Each author should submit a separate form and is responsible for the accuracy and completeness of the submitted information. The form is in six parts.

Identifying information.

The work under consideration for publication.

This section asks for information about the work that you have submitted for publication. The time frame for this reporting is that of the work itself, from the initial conception and planning to the present. The requested information is about resources that you received, either directly or indirectly (via your institution), to enable you to complete the work. Checking "No" means that you did the work without receiving any financial support from any third party -- that is, the work was supported by funds from the same institution that pays your salary and that institution did not receive third-party funds with which to pay you. If you or your institution received funds from a third party to support the work, such as a government granting agency, charitable foundation or commercial sponsor, check "Yes"

1.

2.

Relevant financial activities outside the submitted work.

This section asks about your financial relationships with entities in the bio-medical arena that could be perceived to influence, or that give the appearance of potentially influencing, what you wrote in the submitted work. You should disclose interactions with ANY entity that could be considered broadly relevant to the work. For example, if your article is about testing an epidermal growth factor receptor (EGFR) antagonist in lung cancer, you should report all associations with entities pursuing diagnostic or therapeutic strategies in cancer in general, not just in the area of EGFR or lung cancer.

Report all sources of revenue paid (or promised to be paid) directly to you or your institution on your behalf over the 36 months prior to submission of the work. This should include all monies from sources with relevance to the submitted work, not just monies from the entity that sponsored the research. Please note that your interactions with the work's sponsor that are outside the submitted work should also be listed here. If there is any question, it is usually better to disclose a relationship than not to do so.

3.

For grants you have received for work outside the submitted work, you should disclose support ONLY from entities that could be perceived to be affected financially by the published work, such as drug companies, or foundations supported by entities that could be perceived to have a financial stake in the outcome. Public funding sources, such as government agencies, charitable foundations or academic institutions, need not be disclosed. For example, if a government agency sponsored a study in which you have been involved and drugs were provided by a pharmaceutical company, you need only list the pharmaceutical company.

Intellectual Property.

This section asks about patents and copyrights, whether pending, issued, licensed and/or receiving royalties.

Relationships not covered above.

Use this section to report other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work.

Definitions.

4.

5.

Entity: government agency, foundation, commercial sponsor, academic institution, etc.
Grant: A grant from an entity, generally [but not always] paid to your organization
Personal Fees: Monies paid to you for services rendered, generally honoraria, royalties, or fees for consulting , lectures, speakers bureaus, expert testimony, employment, or other affiliations
Non-Financial Support: Examples include drugs/equipment supplied by the entity, travel paid by the entity, writing assistance, administrative support, etc.

Other: Anything not covered under the previous three boxes
Pending: The patent has been filed but not issued
Issued: The patent has been issued by the agency
Licensed: The patent has been licensed to an entity, whether earning royalties or not
Royalties: Funds are coming in to you or your institution due to your patent

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Section 1. Identifying Information

1. Given Name (First Name) | 2. Surname (Last Name) | 3. Date

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4. Are you the corresponding author? ☑ Yes ☐ No

5. Manuscript Title

Splicing in the pathogenesis, diagnosis and treatment of ciliopathies

6. Manuscript Identifying Number (if you know it)

Section 2. The Work Under Consideration for Publication

Did you or your institution **at any time** receive payment or services from a third party (government, commercial, private foundation, etc.) for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc.)?

Are there any relevant conflicts of interest? ☐ Yes ☑ No

ADD

Section 3. Relevant financial activities outside the submitted work.

Place a check in the appropriate boxes in the table to indicate whether you have financial relationships (regardless of amount of compensation) with entities as described in the instructions. Use one line for each entity; add as many lines as you need by clicking the "Add +" box. You should report relationships that were **present during the 36 months prior to publication**.

Are there any relevant conflicts of interest? ☐ Yes ☑ No

ADD

Section 4. Intellectual Property -- Patents & Copyrights

Do you have any patents, whether planned, pending or issued, broadly relevant to the work? ☐ Yes ☑ No

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☑ No other relationships/conditions/circumstances that present a potential conflict of interest

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