



**HAL**  
open science

## International consensus guideline for reporting transmission electron microscopy results in the diagnosis of primary ciliary dyskinesia (BEAT PCD TEM Criteria)

Amelia Shoemark, Mieke Boon, Christoph Brochhausen, Zuzanna Bukowy-Bieryllo, Maria de Santi, Patricia Goggin, Paul Griffin, Richard Hegele, Robert Hirst, Margaret Leigh, et al.

### ► To cite this version:

Amelia Shoemark, Mieke Boon, Christoph Brochhausen, Zuzanna Bukowy-Bieryllo, Maria de Santi, et al.. International consensus guideline for reporting transmission electron microscopy results in the diagnosis of primary ciliary dyskinesia (BEAT PCD TEM Criteria). *European Respiratory Journal*, 2020, 55 (4), pp.1900725. 10.1183/13993003.00725-2019 . inserm-03791167v2

**HAL Id: inserm-03791167**

**<https://inserm.hal.science/inserm-03791167v2>**

Submitted on 26 Jul 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**HAL**  
open science

## International consensus guideline for reporting transmission electron microscopy results in the diagnosis of primary ciliary dyskinesia (BEAT PCD TEM Criteria)

Amelia Shoemark, Mieke Boon, Christoph Brochhausen, Zuzanna Bukowy-Bieryllo, Maria de Santi, Patricia Goggin, Paul Griffin, Richard Hegele, Robert Hirst, Margaret Leigh, et al.

### ► To cite this version:

Amelia Shoemark, Mieke Boon, Christoph Brochhausen, Zuzanna Bukowy-Bieryllo, Maria de Santi, et al.. International consensus guideline for reporting transmission electron microscopy results in the diagnosis of primary ciliary dyskinesia (BEAT PCD TEM Criteria). *European Respiratory Journal*, 2020, 55 (4), pp.1900725. 10.1183/13993003.00725-2019 . inserm-03791167v2

**HAL Id: inserm-03791167**

**<https://inserm.hal.science/inserm-03791167v2>**

Submitted on 26 Jul 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# International consensus guideline for reporting transmission electron microscopy results in the diagnosis of Primary Ciliary Dyskinesia (BEAT PCD TEM Criteria)

Amelia Shoemark<sup>1,2</sup>, Mieke Boon<sup>3</sup>, Christoph Brochhausen<sup>4</sup>, Zuzanna Bukowy-Bieryllo<sup>5</sup>, Maria Margherita De Santi<sup>6</sup>, Patricia Goggin<sup>7</sup>, Paul Griffin<sup>1,8</sup>, Richard G Hegele<sup>9</sup>, Robert A. Hirst<sup>10</sup>, Margaret W Leigh<sup>11</sup>, Alison Lupton<sup>12</sup>, Karen MacKenney<sup>13</sup>, Heymut Omran<sup>14</sup>, Jean-Claude Pache<sup>15</sup>, Andreia Pinto<sup>16</sup>, Finn P Reinholt<sup>17</sup>, Josef Schroeder<sup>4</sup>, Panayotis Yiallourous<sup>18</sup>, Estelle Escudier<sup>19\*</sup> These authors represent a larger guideline development group acknowledged below.

1. Royal Brompton Hospital, London, UK
2. School of Medicine, University of Dundee, UK
3. Department of Pediatrics, University Hospital Leuven, Belgium
4. Institute of Pathology, University Regensburg, Regensburg, Germany
5. Institute of Human Genetics, Polish Academy of Sciences, Poland
6. Department of Pathology, University Hospital of Siena, Italy.
7. University Hospital Southampton NHS Foundation Trust, Southampton, UK
8. Royal Childrens Hospital, Melbourne, Australia
9. Hospital for Sick Children-University of Toronto, Toronto, Ontario, Canada
10. Department of Respiratory Sciences, University of Leicester, Leicester UK
11. Department of Pediatrics and Marsico Lung Institute, University of North Carolina, Chapel Hill, North Carolina, USA
12. Greater Glasgow and Clyde, Queen Elizabeth University Hospital, Pathology Department, UK
13. NSW Health Pathology, Concord Repatriation General Hospital, Sydney, Australia
14. Department of Pediatrics; University Hospital Muenster; Germany
15. University Hospital of Geneva, Switzerland
16. Instituto de Medicina Molecular, Portugal
17. Oslo University Hospital, Oslo, Norway
18. Medical School, University of Cyprus, Cyprus
19. Sorbonne Université, Faculté de Médecine, INSERM UMR\_S933, (APHP) Assistance Publique Hôpitaux de Paris and CHIC (centre hospitalier intercommunal de Créteil), France

## ABSTRACT

Primary Ciliary Dyskinesia (PCD) is a heterogeneous genetic condition. European and North American diagnostic guidelines recommend transmission electron microscopy (TEM) as one of a combination of tests to confirm a diagnosis. However, there is no definition of what constitutes a defect or consensus on reporting terminology. The aim of this project was to provide an internationally agreed ultrastructural classification for PCD diagnosis by TEM.

A consensus guideline was developed by PCD electron microscopy experts representing 18 centres in 14 countries. An initial meeting and discussion were followed by a Delphi consensus process. The agreed guideline was then tested, modified and retested through exchange of samples and electron micrographs between the 18 diagnostic centres.

The final guideline a) Provides agreed terminology and a definition of class 1 defects which are diagnostic for PCD; b) Identifies class 2 defects which can indicate a diagnosis of PCD in combination with other supporting evidence; c) Describes features which should be included in a ciliary ultrastructure report to assist multidisciplinary diagnosis of PCD d) Defines adequacy of a diagnostic sample.

This tested and externally validated statement provides a clear guideline for the diagnosis of PCD by TEM which can be used to standardise diagnosis internationally.

## INTRODUCTION

Primary Ciliary Dyskinesia (PCD) is a heterogeneous inherited condition affecting 1:10,000 population (1). Symptoms usually begin early in life and include: chronic nasal discharge and wet cough, progressing in childhood to recurrent upper and lower airway infections and eventual bronchiectasis (1). As cilia are also present at the embryonic node defects in nodal cilia may cause abnormalities of left-right laterality determination (situs abnormalities).

Diagnosis of PCD usually relies on a combination of tests, because a single test cannot reliably diagnose all PCD types. These tests may include nasal nitric oxide measurement(2), assessment of ciliary waveform by high speed video microscopy(3), immunofluorescence analysis of ciliary proteins(4), transmission electron microscopy (TEM) and genetic testing ).European and North American diagnostic guidelines differ in recommendations for diagnostic testing although both agree that assessment of ciliary ultrastructure by TEM and/ or bi-allelic mutations in a known PCD gene definitively confirm a PCD diagnosis (5-7). However, there has been considerable heterogeneity among pathology reports describing PCD and interpreting what constitutes a diagnostic defect. Defects of the ciliary axoneme can occur secondary to infection or inflammation of the airway mucosa or in samples which have not been adequately prepared. Differences in reporting and interpretation of findings can lead to misdiagnosis.

In recent years advances in genetic testing and molecular biology have redefined many aspects of PCD, whilst improving understanding of the basic science has further complicated the use of traditional electron microscopy terminology. For example the use of the term 'radial spoke defect' has been used to describe both central complex defects such as those caused by *RSPH4A* mutations and microtubular disarrangement defects caused by *CCDC39* mutations (8-11). In other clinical entities such as renal allograft pathology internationally agreed pathology classification known as 'the Banff criteria' has been shown to significantly improve diagnostic precision, reproducibility and disease outcome (12, 13). The aim of the present project was to provide an internationally agreed ultrastructural classification for the diagnosis of PCD by defining defects diagnostic for PCD, describing features which should be included in a report to assist multidisciplinary diagnosis of PCD and define adequacy of a diagnostic sample.

## METHODS

### Guideline development

The guideline development process is shown in Figure 1. A group of electron microscopy experts (representing 18 centres in 14 countries: Australia, Belgium, Canada, Cyprus, France, Germany, Italy, Norway, Poland, Portugal, Spain, Switzerland, United Kingdom, USA) met in person and via video link to develop a consensus statement for reporting ciliary ultrastructure by TEM at a COST Action BEATPCD meeting. Minutes from this meeting were used to create common themes and identify items for a Delphi consensus survey. The Delphi consensus survey consisted of three rounds with aims to:

- a) Define defects diagnostic for PCD

- b) Describe features which should be included in a report to assist multidisciplinary diagnosis of PCD
- c) Define adequacy of a diagnostic sample

Agreement was considered to be met if > 80% of the participants agreed.

Consultation with the wider BEATPCD community was conducted at the next BEATPCD meeting.

### **Guideline validation**

Once a draft guideline was complete a series of TEM sections were distributed among the group and the guideline was tested and modified for inconsistencies and clarity.

A list of 16 cases representing patients that had a genetically confirmed diagnosis of PCD or conclusively did not have PCD were used for the guideline validation. Four centres were assigned four cases from the following to prepare: *CCDC114*, *CCDC39*, *CCDC40*, *CCNO*, *DNAAF1*, *DNAAF3*, *DNAH5*, *DNAH11*, *DRC1*, *RSPH4A*, cystic fibrosis, healthy volunteer (x2), inadequate sample (x2), unaffected sibling of a patient with PCD. Centres at Southampton (UK), Toronto (Canada), Paris (France) and Muenster (Germany) sectioned and stained 4 grids from 4 cases each. These grids were sent to London where they were quality checked, anonymised, randomised and distributed by an individual who was not part of the consensus group. 17 centres then received 4 grids and 1 centre images. Centres did not receive their own grids.

All 17 centres assessed the 4 grids, each centre using its own technology for making and interpreting images. The 18<sup>th</sup> centre received images. The centres completed a report form based on the consensus statement and returned representative images. The form included class of defect, ultrastructural defect, adequacy of the sample and a summary of key findings. One centre reviewed images taken at the distribution centre in London, rather than grids, due to technical difficulties.

Results of the validation exercise were discussed in a face to face meeting of the group. One additional online survey was distributed to clarify points from this meeting and the guideline was modified accordingly. Results of the guideline surveys and validation can be found in the supplementary information.

Finally images from the cases with *CCDC39*, *DNAAF3*, *DNAH5*, *RSPH4A*, and cystic fibrosis were used to re-assess the modified completed guideline. This validation exercise was completed by all consensus statement authors and 6 additional electron microscopists who were not directly involved in developing the consensus statement.

## **RESULTS**

This guideline describes assessment and reporting of ciliated epithelial samples fixed in glutaraldehyde, dehydrated, embedded in resin and stained with heavy metals (lead citrate and uranyl acetate or equivalent).

**Normal ultrastructure of respiratory cilia.** The consensus group identified that it is crucial for the observer to be familiar with the appearance of normal ciliary ultrastructure and PCD defects from extensive samples using local sampling, processing and visualisation methods before embarking on diagnostic testing for PCD. Figure 2 shows the normal ultrastructure of a healthy respiratory cilium. The

axoneme of motile cilia is composed of the well-known “9+2” structure: 9 peripheral microtubule doublets surround the central pair of single microtubules. Each microtubular doublet is connected with the central pair by radial spokes, and neighboring MT doublets are connected by the nexin dynein regulatory complex (N-DRC). The outer microtubular doublets contain regularly repeating hook shaped structures known as outer dynein arms (ODA) and inner dynein arms (IDA), which are responsible for the generation of ciliary motion through ATPase activity. Essential units repeat along the axoneme approximately every 96nm. Each doublet contains in a 96 nm repetition 4 double headed outer dynein arms (ODAs), 6 different single headed and 1 double headed inner dynein arm (IDA) isoform, 1 N-DRC and 3 radial spokes (14-17). Differences in the repetition of these units and their density results in differing contrasts on TEM. The electron dense ODA is easier to visualise than the less dense IDA and complex-DRC in a 70-100nm TEM section, because within the slice more ODA dynein heavy chains are present. Ultrastructure at the tip and base of the cilium is different and this should be considered when assessing for PCD defects.

### **Class 1 and class 2 defects, terminology, adequacy of sampling and consensus definitions for the diagnosis of PCD by TEM**

The expert consensus group identified two classes of PCD TEM diagnostic defects: Class 1 defects which are considered ‘hallmark’ defects confirming a diagnosis in a patient with symptoms of the condition and class 2 defects which indicate a diagnosis of PCD in a patient with clinical symptoms of the condition if consistent across more than one sample or after cell culture AND consistent with other test results. The two classes of defects are shown in table 1 and defined in the text. The group made distinction between the two classes of defect as class one defects are diagnostic whereas class two defects can be more difficult to recognise and can be similar to secondary defects.

**Table 1: Summary of class 1 and class 2 defects for the ultrastructural diagnosis of primary ciliary dyskinesia**

#### **Class 1 defects: Hallmark diagnostic defects**

- Outer dynein arm defect
- Outer and inner dynein arm defect
- Microtubular disorganisation and inner dynein arm defect

#### **Class 2 defects: Indicate a PCD diagnosis with other supporting evidence**

- Central complex defect
- Mislocalisation of basal bodies with few or no cilia
- Microtubular disorganisation defect with inner dynein arm present
- Outer dynein arm absence from 25%-50% cross sections
- Combined inner and outer dynein arm absence from 25-50% cross sections

#### Class 1 defects (hallmark defects)

Description: Class 1 defects *are diagnostic of PCD in a patient with clinical symptoms of the condition.*

Sample adequacy: Class 1 defects are confirmed following assessment of >50 axonemes in transverse section from several cells. Care should be taken to assess cross sections from both proximal (in the

region of the microvili) and distal regions of the axoneme. Dynein arms should be assessed in cross sections with clear structural features and an intact ciliary membrane. Microtubular arrangement may be assessed in a larger number of cross sections with an intact membrane in which the dynein arms may not be clear. Examples of class 1 defects are shown in Figure 3.

#### *Outer dynein arm (ODA) defect:*

Definition: Absence of the whole or larger part of the outer dynein arm structure from the majority of ( $\geq 5$ ) microtubular doublets in the majority (>50%) of cilia cross sections

The defects arise from *mutations in genes coding for ODA structural proteins (e.g DNAH5) and ODA docking proteins (e.g. CCDC151)*. IF analyses usually show an absence of the outer dynein heavy chain protein DNAH5.

#### *Outer and inner dynein arm (ODA+IDA) defect:*

Definition: Absence of the whole or larger part of the outer dynein arm structure from the majority of ( $\geq 5$ ) microtubular doublets in the majority (>50%) of cilia cross sections AND absence of the whole or larger part of the inner dynein arm structure from the majority of ( $\geq 7$ ) microtubular doublets in the majority (>50%) of cilia cross sections.

***If the number of inner dynein arms cannot be accurately determined samples should be reported as outer dynein arm defect (+/- inner dynein arm defect)***

These defects often arise from *mutations in dynein assembly genes*. IF analyses show absence of proteins such as DNAH5 and DNALI1.

#### *Microtubular disorganisation and inner dynein arm defect:*

Definition: Disruption of the 9+2 symmetry of the microtubules in >25% cross sections combined with absence of the whole or larger part of the inner dynein arm structure from the majority of (> 7) microtubular doublets in the majority (>50%) of cilia axonemes visualised in cross section.

The defects almost always arise from *CCDC39 or CCDC40 mutations*.<sup>7</sup> IF analyses show in those cases absence of N-DRC proteins such as GAS8 and inner dynein arm proteins such as DNALI1.

#### Class 2 defects

Description: Class 2 defects *can indicate a diagnosis of PCD in a patient with clinical symptoms of the condition if consistent across more than one sample AND consistent with other results such as those from immunofluorescence, high speed video microscopy or genetic analysis*. They may require assessment of more ciliary axonemes than class 1 defects. Care should be taken to assess cross sections from several cells and both proximal (in the region of the microvili) and distal regions of the axoneme. Dynein arms should be assessed in cross sections with clear structural features and an intact ciliary membrane. Microtubular arrangement may be assessed in a larger number of cross sections with an

intact membrane in which the dynein arms may not be clear. Examples of class 2 defects are shown in Figure 4.

#### *Central complex defect:*

Description: Central complex defects consist of a proportion of normal cross sections and consistent cross sections with one or both of the central microtubule(s) absent (usually >20%) Occasional double central pairs or translocation of the outer microtubule to the central region (8+1) may be seen. Translocation typically occurs approaching the ciliary tip. In longitudinal sections, Intermittent or complete loss of the central pair, or transposition of an outer doublet may also be seen. Some microtubular disorganisation is often seen. These TEM findings are reported to be associated with genetic defects of the radial spoke components (e.g. *RSPH4A*, *RSPH1*, *RSPH9* and *DNAJB13*)(18-20) which can be confirmed by immunofluorescence for radial spoke head proteins. In some cases high speed video microscopy can show circling of cilia when observed from above.

#### *Mislocalisation of basal bodies with few or no cilia:*

Description: Findings are typically of no or very few ciliary cross sections in combination with failure of the majority of basal bodies to dock at the apical surface of the cell, meaning they are consistently seen within the cytoplasm. These findings are associated with genetic variants in *CCNO* and *MCIDAS* and by high speed video microscopy only a few cilia are seen per cell (21, 22). The genetic defects result in reduced generation of multiple motile cilia. In the rare cilia that are visualised the ultrastructure of the axoneme in *CCNO* cases can be normal but in individuals with *MCIDAS* mutations can lack ODAs.

#### *Microtubular disorganisation:*

Description: Disruption of the 9+2 symmetry of the microtubules in cross sections consistently throughout an otherwise healthy sample. The absence of the dynein regulatory complex/nexin link is sometimes noted. Usually the majority of cross sections have normal ultrastructure with outer and inner dynein arms present. These findings might be associated with mutations in *CCDC65*, *DRC1* and *GAS8* encoding N-DRC proteins (23-25). Subtle beat pattern abnormalities including fast or disrupted beat pattern and an absence of N-DRC proteins such as *GAS8* are observed by immunofluorescence in these cases. The inner dynein arm is present in >50% of cross sections, distinguishing this from the class1 microtubular disorganisation and inner dynein arm defect.

#### *Outer dynein arm absence from 25%-50% cross sections*

This definition is the same as for a class 1 outer dynein arm defect except dynein arm(s) are absent in a minority of cilia and numerous normal ciliary cross sections are present. A pattern to the defect may be observed e.g. In individuals with recessive *DNAH9* mutations the distal ciliary axonemes lack ODAs whereas the proximal ciliary axonemes are normal. (26, 27). Where a pattern to the defect is observed by TEM immunofluorescence may also show a partial absence of *DNAH5*.



### *Combined inner and outer dynein arm absence from 25-50% cross sections*

This definition is the same as for a class 1 outer and inner dynein arm defect except for the dynein arms being absent in a minority of cilia and numerous normal ciliary cross sections are present. Some static cilia may be present by high speed video microscopy and immunofluorescence may or may not show a partial absence of DNAH5 and DNALI1. This is seen for example in patients with the missense mutation His154Pro in *CCDC103* (28, 29).

### **The transmission electron microscopy cilia report**

A TEM report should conform to general considerations for good practice in pathology reporting:

Reports should be clear and accurate and the overall result or conclusion must be clearly visible. Patients must be identified on reports by at least two unique items of information e.g. full name and date of birth. It must be stated if the testing is incomplete and/or where the minimum quality is not achieved. For PCD testing **it should be made clear to the person receiving the report that normal ultrastructure does not exclude a diagnosis.**

Table 2 describes items agreed by the expert group as essential and desirable for an electron microscopy report in the diagnosis of PCD

### **Table 2: Items to include in a TEM report for the diagnosis of Primary Ciliary Dyskinesia**

#### **Essential items to be included in a TEM report for PCD**

- Source of the sample (e.g. nasal brushing)
- Adequacy of the sample
- Number of cross sections assessed
- % abnormal cilia (class 1 and class 2 defects)
- Consistency of a defect across several cells
- One sentence summary of key findings (including class 1 or 2 defect if present)

#### **Additional items to consider enhancing a report**

- Orientation/ alignment of the basal body or central pair of microtubules
- Number of cells assessed
- Blebs/membrane swelling/membrane condition
- Presence of compound cilia (more than one axoneme within a membrane)
- Preservation of the sample
- % cilia with other defects
- Presence of shortened or truncated ODA projections
- Evidence of inflammation
- Evidence of bacteria

## **Summary of guideline development survey results (Full results are shown in the supplementary information)**

In round 1 consensus was met on the 3 hallmark defects shown in table 1 and participants suggested definitions. >80% agreed that the following were class 2 defects: Central complex defect, Mislocalisation of basal bodies with few or no cilia, Outer dynein arm absence from 25%-50% cross sections, Combined inner and outer dynein arm absence from 25-50% cross sections. Microtubular disorganisation was added to this list following the validation exercise. Items to include in a report were also agreed (table 2).

The consensus group did not reach consensus on the following items as class 1 or 2 defects and inclusion or exclusion of these should be at the discretion of the observer as evidence becomes available: Isolated inner dynein arm defects (30), ODA loss <25% cross sections, ODA and IDA loss <25% cross sections, orientation defect (31-33), ciliary length defects. Future research should focus on the relevance of these defects and the guideline should be updated accordingly.

In round 2 and 3 definitions of hallmark defects were agreed along with the criteria for sample adequacy, i.e. >50 cross sections with intact ciliary membranes.

## **Summary of guideline validation results**

Two rounds of validation were conducted. There was 100% participation (18 centres) in this activity. In round one there were no false positive diagnoses (i.e. no non-PCD sample was classed as having a Class 1 Hallmark PCD defect). 17/68 (25%) sections were described as insufficient or inadequate for assessment. These included 100% returns on a CCNO case and 75% returns for a sample included as an inadequate sample. 25/25 cases (100%) were correctly identified as a class 1 hallmark defect. However 8/25 (32%) returns recorded an incorrect name of the class 1 defect. 2 cases were described as having a class 1 defect when they had a class 2 defect or normal ultrastructure. 5/6 cases were correctly identified as class 2 defects. 4 normal ultrastructure cases were erroneously identified as a class 2 defect.

The guideline was modified and in round two there were no false positive diagnoses (i.e. no non-PCD sample was classed as having a Class 1 hallmark PCD defect) and normal ultrastructure identified by 18/18 centres. There were no false negatives. There was 100% correct identification as a class 1 hallmark defect. These included 18/18 correctly identified MTD + IDA, 17/18 ODA (1 judged as ODA+IDA) and 17/18 ODA+IDA (1 judged ODA). There was 100% correct identification as a class 2 defect. 15/18 correctly identified a central complex defect whereas 3 judged this as MTD. This stresses the importance of the additional evidence for class 2 defects. 94% of reviewers gave a correct ultrastructural defect according to the genetic diagnosis. Agreement between observers was kappa 0.76 -1.0 (where 0= no agreement and 1= complete agreement).

For external validation a further 6 electron microscopists were given the guideline to read and then completed the image validation. All six observers identified the correct ultrastructural defect for all cases.

## **Discussion**

Next generation sequencing and molecular advances have resulted in fast paced improvement of PCD diagnosis over recent years. TEM is valuable to assert and/or to confirm a diagnosis of PCD and as a guide for genetic testing, as there is a clear link between the ultrastructural defect and the group of affected genes. This is useful especially when variants of unknown significance are identified. Pathogenic mutations in known PCD genes currently only account for 65-75% cases of PCD confirmed by TEM. A table of known TEM phenotypes according to genotype is available in the supplementary information. We present the first internationally agreed guideline for reporting of ciliary biopsies for the diagnosis of PCD. This guideline is designed to be a flexible formulation, it will require updating and evolution as new evidence becomes available. These recommendations are minimum requirements and professional judgement and experience is of paramount importance in many circumstances.

### **PCD with normal ultrastructure**

It has been shown repeatedly that the diagnostic accuracy of TEM is not sufficient to exclude a diagnosis as up to 30% of all PCD cases are described as having normal or near normal ciliary ultrastructure (34-36). This includes the large dynein heavy chain *DNAH11* which accounts for a substantial number of cases (37, 38). In another group of patients subtle changes to ultrastructure can be seen but alone these are non-diagnostic for example defects in *HYDIN* which affect the c2b projection of the central pair (39). The current statement introduces the concept of class 2 defects to address limitations with the identification of subtle changes. Class 2 defects support a diagnosis but should not be used without other diagnostic tests. Many of these class 2 defect changes are secondary to the causative molecular defect, for example radial spoke head gene defects result in secondary loss of the central pair (19). It must be stressed that some class 2 defects, if not confirmed in a second sample or after cell culture, may also be the result of non-PCD related causes.

### **Limitations of electron microscopy in the diagnosis of PCD**

Initial validation of the guideline (supplementary information) revealed that smaller inadequate samples were most likely to lead to a misdiagnosis. It was also clear that individual processing techniques and familiarity with local appearance of cilia is paramount to interpretation. Due to differences in equipment availability and local procedures it is not currently possible to standardise all methodology in sample processing and visualisation. In the future a consensus methodology could be developed. However we have addressed methodological differences by recommending that extensive normal and PCD samples are visualised using local techniques before a diagnostic sample is interpreted.

Secondary ultrastructural defects in cilia can be caused by acute or chronic respiratory infections, inflammatory respiratory disease (i.e. asthma), environmental and demographic factors (smoking, pollution, age) or sample handling (40). It is common to observe secondary defects in samples examined by TEM, for example microtubular defects that have been observed in up to 10% of healthy controls (41, 42). The appearance of secondary defects can overlap with the appearance of positive PCD cases e.g., microtubular disorganisation, central pair abnormalities. Misinterpretation of these defects can be prevented by taking care to avoid sampling when the patient is unwell, use of cell culture of respiratory epithelium (air-liquid interface or spheroid cultures) to remove conditions causing the secondary effects, consideration of the health of individual cells in the sample and by repeating the biopsy(43, 44). To ensure that occasional secondary defects in the sample do not bias interpretation, we

recommend assessment of at least 50 axonemes in transverse section from a number of different cells. We recommend dynein arms should be assessed in cross sections with clear structural features and intact ciliary membranes. Microtubular arrangement may be assessed in a larger number of cross sections with intact membranes in which the dynein arms are not clear although unhealthy cells or compound cilia should be avoided. For class 2 defects we recommend assessment of more than one sample or following cell culture. Paramount is the consistency of a class 2 defect with results of other tests such as high speed video, immunofluorescence analysis, nasal nitric oxide measurement and genotyping.

### **Summary**

Performance (sensitivity and specificity) of TEM for PCD diagnosis is difficult to accurately assess when there is no agreed diagnostic criteria. This consensus statement is tested and validated and provides a clear guideline for the diagnosis of PCD by TEM which can be used to standardise diagnosis internationally.

### **Supplementary information:**

Results of consensus surveys

Results of validation activities

Example cases and reports for hallmark and class 2 defects

Table of genotype associations with TEM phenotype

### **Figures**

Figure 1: Figure 1: Flow chart outlining the methodology for development of the TEM consensus guideline. Results of Delphi surveys and case reviews can be found in the supplementary information

Figure 2: Electron micrographs showing normal ciliary ultrastructure in cross section A) The core of the ciliary axoneme with 9+2 microtubular arrangement. Black arrows depict the outer dynein arms (ODA) and black arrows with white outline the inner dynein arm (IDA). B) Cross sections from the tip of the cilium with single microtubules (indicated by grey arrows) are a normal part of ciliary ultrastructure and should not be assessed for diagnosis of class 1 or class 2 defects. C) Ultrastructurally normal ciliary cross sections from the base of the axoneme. White arrow indicates that these cross sections have no central pair. Microtubular doubles are often linked to the ciliary membrane with Y shaped linker D) Diagrammatic representation of ciliary cross sections at the tip, central axoneme and base

Figure 3: Electron micrographs and diagrams of class 1 ultrastructural defects in cross section. Left panel; Outer dynein arm defect, central panel outer and inner dynein arm defect, right panel microtubular disorganisation and inner dynein arm defect. Please note not all arms are completely missing. Although the curved hook structure of the ODA is not present in its entirety the white star indicates some remaining proximal structure likely to be the docking complex. Class 1 defects are diagnostic of PCD in patients with symptoms of the condition. Class 1 defects are confirmed following assessment of >50 axonemes in transverse section. Dynein arms should be assessed in cross sections

with clear structural features and an intact ciliary membrane as depicted. Microtubular arrangement may be assessed in a larger number of cross sections with an intact membrane in which the dynein arms may not be clear.

Figure 4: Electron micrographs of class 2 ultrastructural defects. Left panel Central complex defect with features A-D demonstrating, lack of central pair, transposed outer microtubular doublet, single central tubule and double central pair. Micrograph from an individual with an RSPH4A mutation. Right panel Mislocalisation of basal bodies with few or no cilia from an individual with a CCNO mutation

One representative lead author (usually the individual assessing and reporting the TEM and representing the opinions of each centre by completing the Delphi survey) per centre has been included in the authorship of this report. However, several additional experts have attended meetings, commented on this consensus statement and contributed their expert opinion and technical skills during its development. We would like to thank: Sverre-Henning Brorson, Tom Burgoyne, Suzanne Crowley, Chris O'Callaghan, Mellisa Dixon, Regan Doherty, Catherine Faucon, Paul French, Raffaella Guazzo, Yew Meng Heng, Claire Hogg, Merete Helgesen, Louise Hughes, Martine Jaspers, Panayiotis Kouis, Kyriacos Kyriacos, Jane Lucas, Virginia Mancini, Paul Martinello, Rana Mitri, Elena Moretti, José Moura Nunes, Anton Page, Phil Robinson, Anna Rowinska, Andrew Rutman, Heiko Siegmund, Jennifer Sweeney, James Thompson, and Valerie Vlaeminck.

In addition we are especially grateful to Farheen Dauvohra for anonymising and randomising sections and collating data for the validation of this guideline

This project was conducted as part of BEAT-PCD, a network of multidisciplinary researchers and clinicians funded by EU COST Action (BM1407)

## References

1. Lucas JS, Burgess A, Mitchison HM, Moya E, Williamson M, Hogg C. Diagnosis and management of primary ciliary dyskinesia. *Arch Dis Child*. 2014;99(9):850-6.
2. Collins SA, Gove K, Walker W, Lucas JS. Nasal nitric oxide screening for primary ciliary dyskinesia: systematic review and meta-analysis. *Eur Respir J*. 2014;44(6):1589-99.
3. Rubbo B, Shoemark A, Jackson CL, Hirst R, Thompson J, Hayes J, et al. Accuracy of High-Speed Video Analysis to Diagnose Primary Ciliary Dyskinesia. *Chest*. 2019.
4. Shoemark A, Frost E, Dixon M, Ollosson S, Kilpin K, Patel M, et al. Accuracy of Immunofluorescence in the Diagnosis of Primary Ciliary Dyskinesia. *Am J Respir Crit Care Med*. 2017;196(1):94-101.
5. Shapiro AJ, Davis SD, Polineni D, Manion M, Rosenfeld M, Dell SD, et al. Diagnosis of Primary Ciliary Dyskinesia. An Official American Thoracic Society Clinical Practice Guideline. *Am J Respir Crit Care Med*. 2018;197(12):e24-e39.
6. Lucas JS, Barbato A, Collins SA, Goutaki M, Behan L, Caudri D, et al. European Respiratory Society guidelines for the diagnosis of primary ciliary dyskinesia. *Eur Respir J*. 2017;49(1).

7. Shoemark A, Dell S, Shapiro A, Lucas JS. ERS and ATS diagnostic guidelines for primary ciliary dyskinesia: similarities and differences in approach to diagnosis. *Eur Respir J*. 2019;54(3).
8. Sturgess JM, Chao J, Turner JA. Transposition of ciliary microtubules: another cause of impaired ciliary motility. *N Engl J Med*. 1980;303(6):318-22.
9. Sturgess JM, Chao J, Wong J, Aspin N, Turner JA. Cilia with defective radial spokes: a cause of human respiratory disease. *N Engl J Med*. 1979;300(2):53-6.
10. Frommer A, Hjeij R, Loges NT, Edelbusch C, Jahnke C, Raidt J, et al. Immunofluorescence Analysis and Diagnosis of Primary Ciliary Dyskinesia with Radial Spoke Defects. *Am J Respir Cell Mol Biol*. 2015;53(4):563-73.
11. Antony D, Becker-Heck A, Zariwala MA, Schmidts M, Onoufriadis A, Forouhan M, et al. Mutations in *CCDC39* and *CCDC40* are the major cause of primary ciliary dyskinesia with axonemal disorganization and absent inner dynein arms. *Hum Mutat*. 2013;34(3):462-72.
12. Solez K, Axelsen RA, Benediktsson H, Burdick JF, Cohen AH, Colvin RB, et al. International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. *Kidney Int*. 1993;44(2):411-22.
13. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, et al. The Banff 97 working classification of renal allograft pathology. *Kidney International*. 1999;55(2):713-23.
14. Kurkowiak M, Zietkiewicz E, Witt M. Recent advances in primary ciliary dyskinesia genetics. *J Med Genet*. 2015;52(1):1-9.
15. Afzelius BA. A human syndrome caused by immotile cilia. *Science*. 1976;193(4250):317-9.
16. Burgoyne T, Dixon M, Luther P, Hogg C, Shoemark A. Generation of a three-dimensional ultrastructural model of human respiratory cilia. *Am J Respir Cell Mol Biol*. 2012;47(6):800-6.
17. Satir P, Christensen ST. Structure and function of mammalian cilia. *Histochem Cell Biol*. 2008;129(6):687-93.
18. Onoufriadis A, Shoemark A, Schmidts M, Patel M, Jimenez G, Liu H, et al. Targeted NGS gene panel identifies mutations in *RSPH1* causing primary ciliary dyskinesia and a common mechanism for ciliary central pair agenesis due to radial spoke defects. *Hum Mol Genet*. 2014;23(13):3362-74.
19. Castleman VH, Romio L, Chodhari R, Hirst RA, de Castro SC, Parker KA, et al. Mutations in radial spoke head protein genes *RSPH9* and *RSPH4A* cause primary ciliary dyskinesia with central-microtubular-pair abnormalities. *Am J Hum Genet*. 2009;84(2):197-209.
20. El Khouri E, Thomas L, Jeanson L, Bequignon E, Vallette B, Duquesnoy P, et al. Mutations in *DNAJB13*, Encoding an HSP40 Family Member, Cause Primary Ciliary Dyskinesia and Male Infertility. *Am J Hum Genet*. 2016;99(2):489-500.
21. Boon M, Wallmeier J, Ma L, Loges NT, Jaspers M, Olbrich H, et al. *MCIDAS* mutations result in a mucociliary clearance disorder with reduced generation of multiple motile cilia. *Nat Commun*. 2014;5:4418.
22. Wallmeier J, Al-Mutairi DA, Chen CT, Loges NT, Pennekamp P, Menchen T, et al. Mutations in *CCNO* result in congenital mucociliary clearance disorder with reduced generation of multiple motile cilia. *Nat Genet*. 2014;46(6):646-51.
23. Olbrich H, Cremers C, Loges NT, Werner C, Nielsen KG, Marthin JK, et al. Loss-of-Function *GAS8* Mutations Cause Primary Ciliary Dyskinesia and Disrupt the Nexin-Dynein Regulatory Complex. *Am J Hum Genet*. 2015;97(4):546-54.
24. Horani A, Brody SL, Ferkol TW, Shoseyov D, Wasserman MG, Ta-shma A, et al. *CCDC65* mutation causes primary ciliary dyskinesia with normal ultrastructure and hyperkinetic cilia. *PLoS One*. 2013;8(8):e72299.
25. Wirschell M, Olbrich H, Werner C, Tritschler D, Bower R, Sale WS, et al. The nexin-dynein regulatory complex subunit *DRC1* is essential for motile cilia function in algae and humans. *Nat Genet*. 2013;45(3):262-8.

26. Fassad MR, Shoemark A, Legendre M, Hirst RA, Koll F, le Borgne P, et al. Mutations in Outer Dynein Arm Heavy Chain DNAH9 Cause Motile Cilia Defects and Situs Inversus. *Am J Hum Genet.* 2018;103(6):984-94.
27. Loges NT, Antony D, Maver A, Deardorff MA, Gulec EY, Gezdirici A, et al. Recessive DNAH9 Loss-of-Function Mutations Cause Laterality Defects and Subtle Respiratory Ciliary-Beating Defects. *Am J Hum Genet.* 2018;103(6):995-1008.
28. Shoemark A, Moya E, Hirst RA, Patel MP, Robson EA, Hayward J, et al. High prevalence of CCDC103 p.His154Pro mutation causing primary ciliary dyskinesia disrupts protein oligomerisation and is associated with normal diagnostic investigations. *Thorax.* 2017.
29. Panizzi JR, Becker-Heck A, Castleman VH, Al-Mutairi DA, Liu Y, Loges NT, et al. CCDC103 mutations cause primary ciliary dyskinesia by disrupting assembly of ciliary dynein arms. *Nat Genet.* 2012;44(6):714-9.
30. O'Callaghan C, Rutman A, Williams GM, Hirst RA. Inner dynein arm defects causing primary ciliary dyskinesia: repeat testing required. *Eur Respir J.* 2011;38(3):603-7.
31. Bukowy-Bieryllo Z, Zietkiewicz E, Loges NT, Wittmer M, Geremek M, Olbrich H, et al. RPGR mutations might cause reduced orientation of respiratory cilia. *Pediatr Pulmonol.* 2013;48(4):352-63.
32. Jorissen M, Willems T. The secondary nature of ciliary (dis)orientation in secondary and primary ciliary dyskinesia. *Acta Otolaryngol.* 2004;124(4):527-31.
33. Rutman A, Cullinan P, Woodhead M, Cole PJ, Wilson R. Ciliary disorientation: a possible variant of primary ciliary dyskinesia. *Thorax.* 1993;48(7):770-1.
34. Boon M, Smits A, Cuppens H, Jaspers M, Proesmans M, Dupont LJ, et al. Primary ciliary dyskinesia: critical evaluation of clinical symptoms and diagnosis in patients with normal and abnormal ultrastructure. *Orphanet J Rare Dis.* 2014;9:11.
35. Knowles MR, Daniels LA, Davis SD, Zariwala MA, Leigh MW. Primary ciliary dyskinesia. Recent advances in diagnostics, genetics, and characterization of clinical disease. *Am J Respir Crit Care Med.* 2013;188(8):913-22.
36. Kouis P, Yiallourous PK, Middleton N, Evans JS, Kyriacou K, Papatheodorou SI. Prevalence of primary ciliary dyskinesia in consecutive referrals of suspect cases and the transmission electron microscopy detection rate: a systematic review and meta-analysis. *Pediatr Res.* 2017;81(3):398-405.
37. Bartoloni L, Blouin JL, Pan Y, Gehrig C, Maiti AK, Scamuffa N, et al. Mutations in the DNAH11 (axonemal heavy chain dynein type 11) gene cause one form of situs inversus totalis and most likely primary ciliary dyskinesia. *Proc Natl Acad Sci U S A.* 2002;99(16):10282-6.
38. Knowles MR, Leigh MW, Carson JL, Davis SD, Dell SD, Ferkol TW, et al. Mutations of DNAH11 in patients with primary ciliary dyskinesia with normal ciliary ultrastructure. *Thorax.* 2012;67(5):433-41.
39. Olbrich H, Schmidts M, Werner C, Onoufriadis A, Loges NT, Raidt J, et al. Recessive HYDIN mutations cause primary ciliary dyskinesia without randomization of left-right body asymmetry. *Am J Hum Genet.* 2012;91(4):672-84.
40. Dixon M, Shoemark A. Secondary defects detected by transmission electron microscopy in primary ciliary dyskinesia diagnostics. *Ultrastruct Pathol.* 2017;41(6):390-8.
41. de Longh RU, Rutland J. Ciliary defects in healthy subjects, bronchiectasis, and primary ciliary dyskinesia. *Am J Respir Crit Care Med.* 1995;151(5):1559-67.
42. Rossman CM, Lee RM, Forrest JB, Newhouse MT. Nasal cilia in normal man, primary ciliary dyskinesia and other respiratory diseases: analysis of motility and ultrastructure. *Eur J Respir Dis Suppl.* 1983;127:64-70.
43. Jorissen M, Willems T, Van der Schueren B, Verbeken E. Secondary ciliary dyskinesia is absent after ciliogenesis in culture. *Acta Otorhinolaryngol Belg.* 2000;54(3):333-42.

44. Hirst RA, Jackson CL, Coles JL, Williams G, Rutman A, Goggin PM, et al. Culture of primary ciliary dyskinesia epithelial cells at air-liquid interface can alter ciliary phenotype but remains a robust and informative diagnostic aid. *PLoS One*. 2014;9(2):e89675.



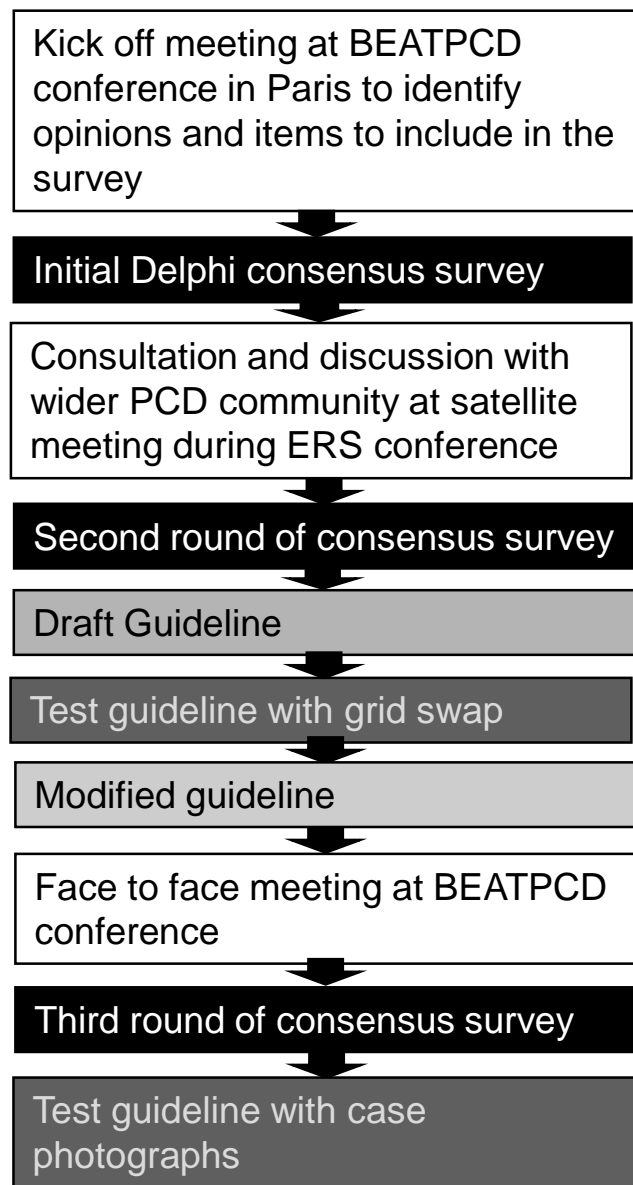


Figure1: Guideline development methodology

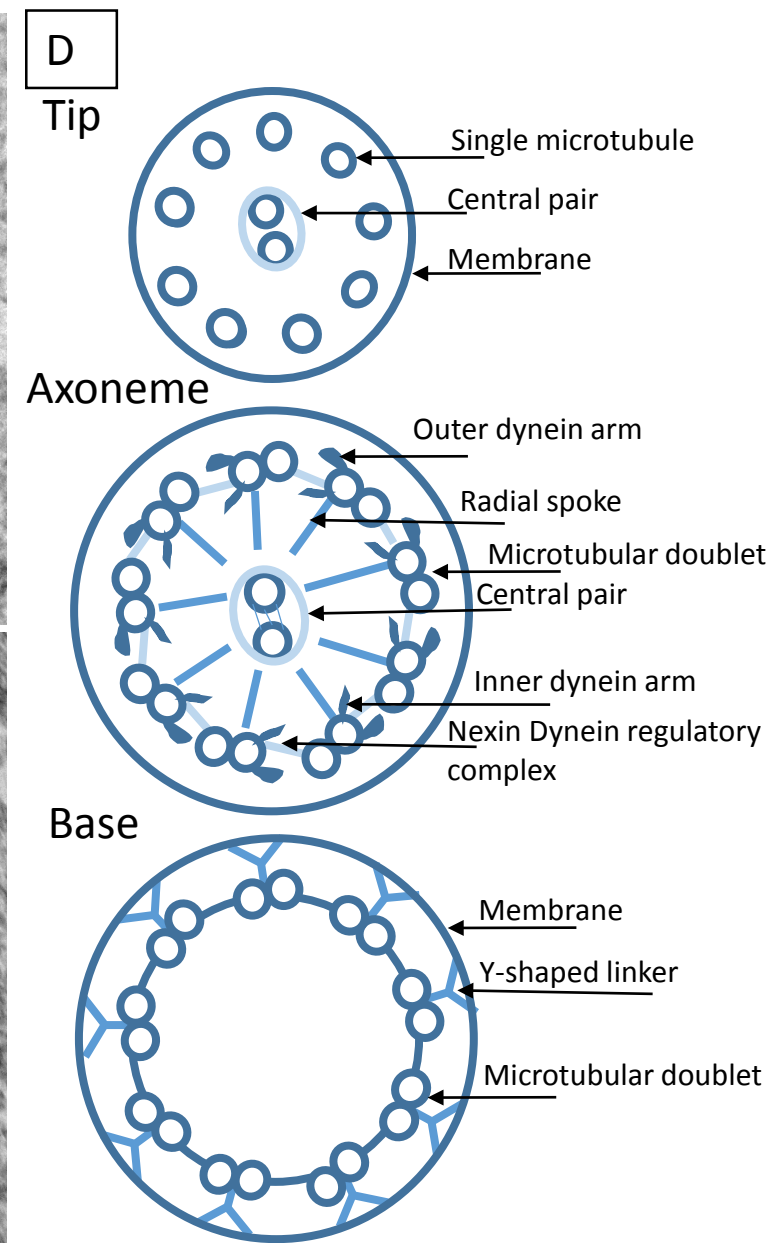
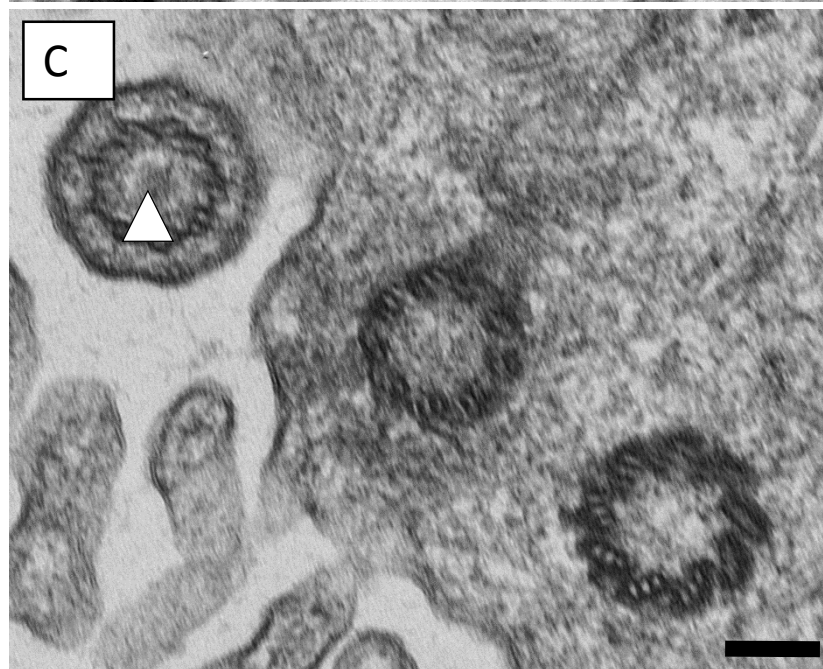
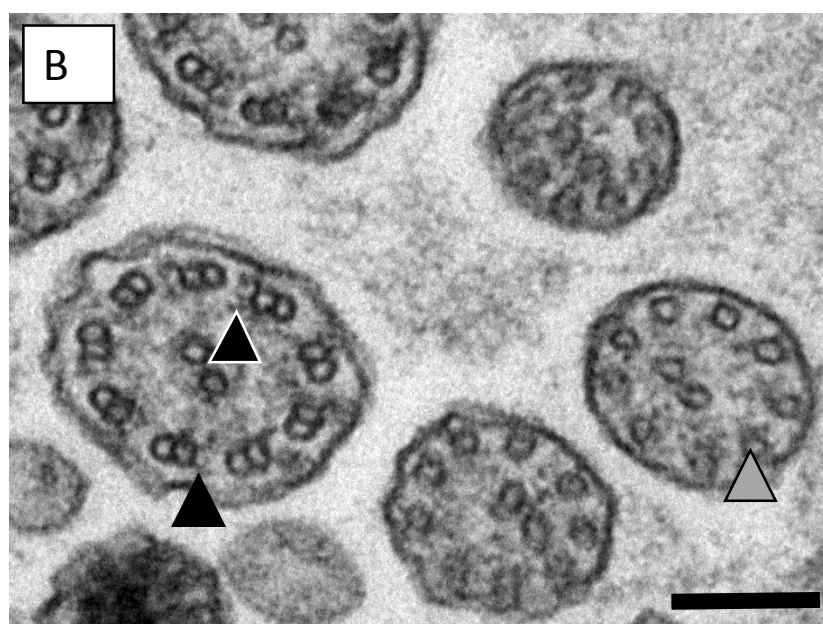
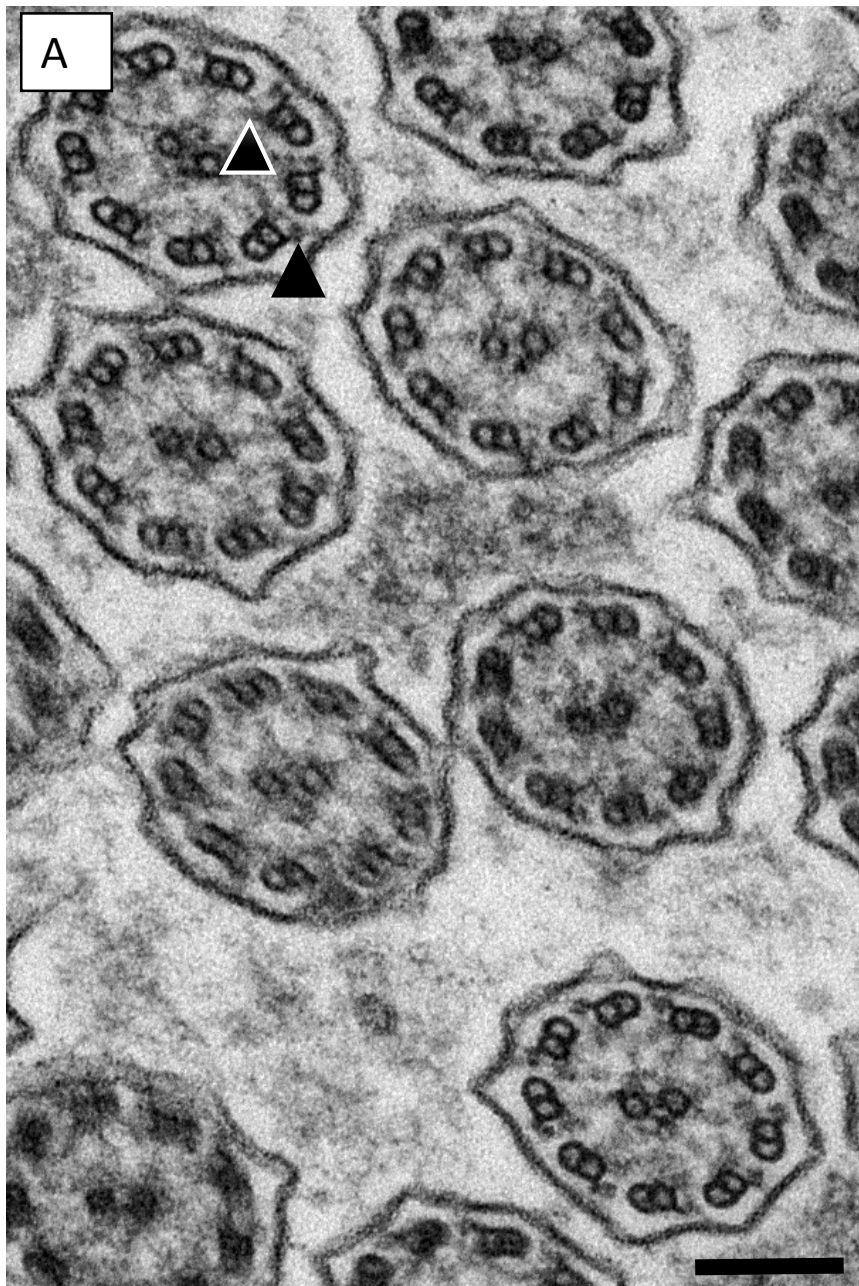
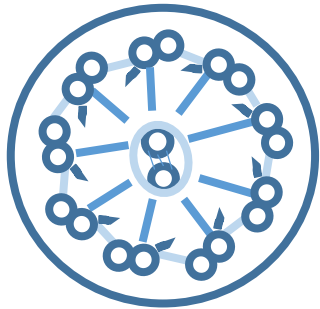
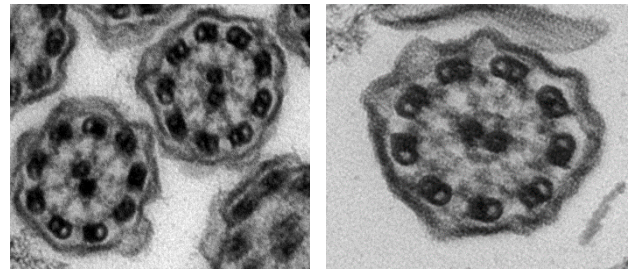
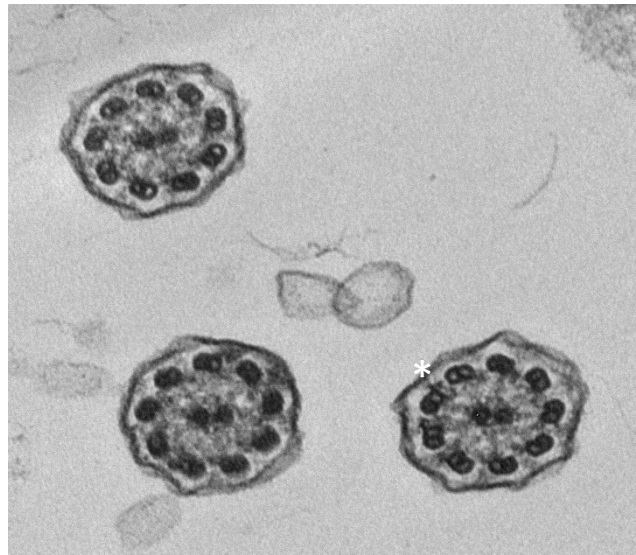
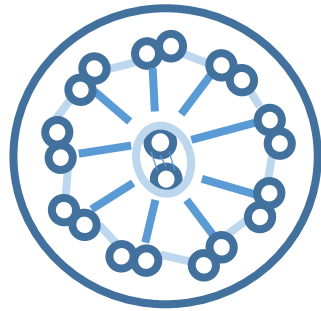
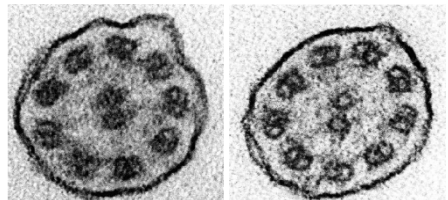
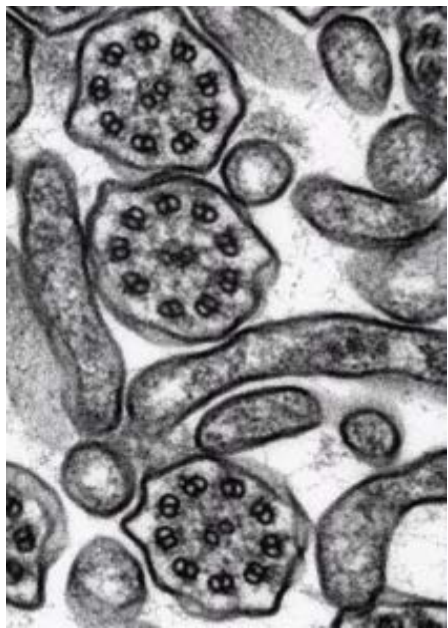


Figure 2: Normal ciliary ultrastructure

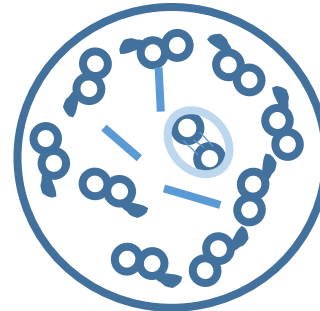
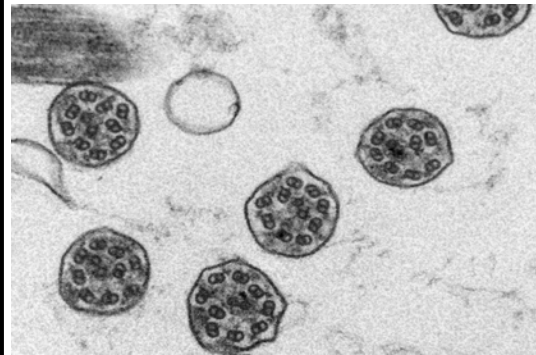
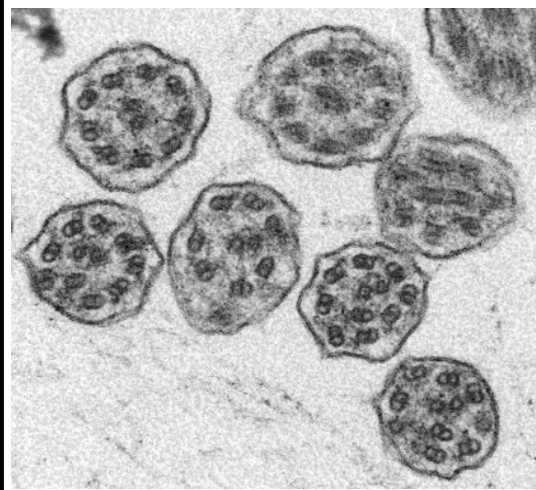




Outer dynein arm defect

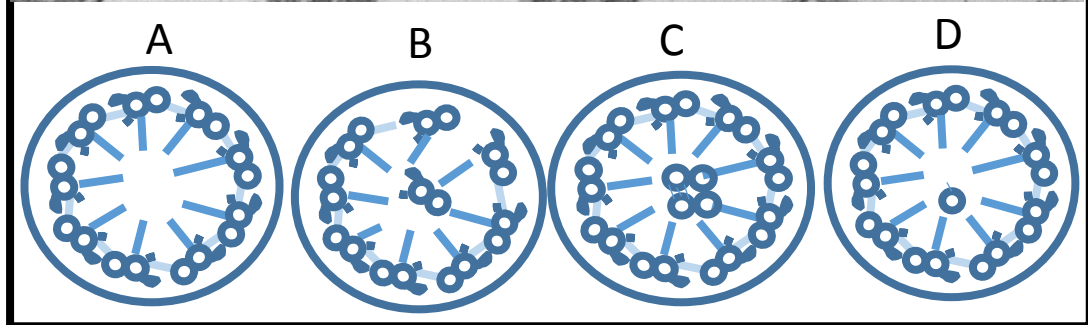
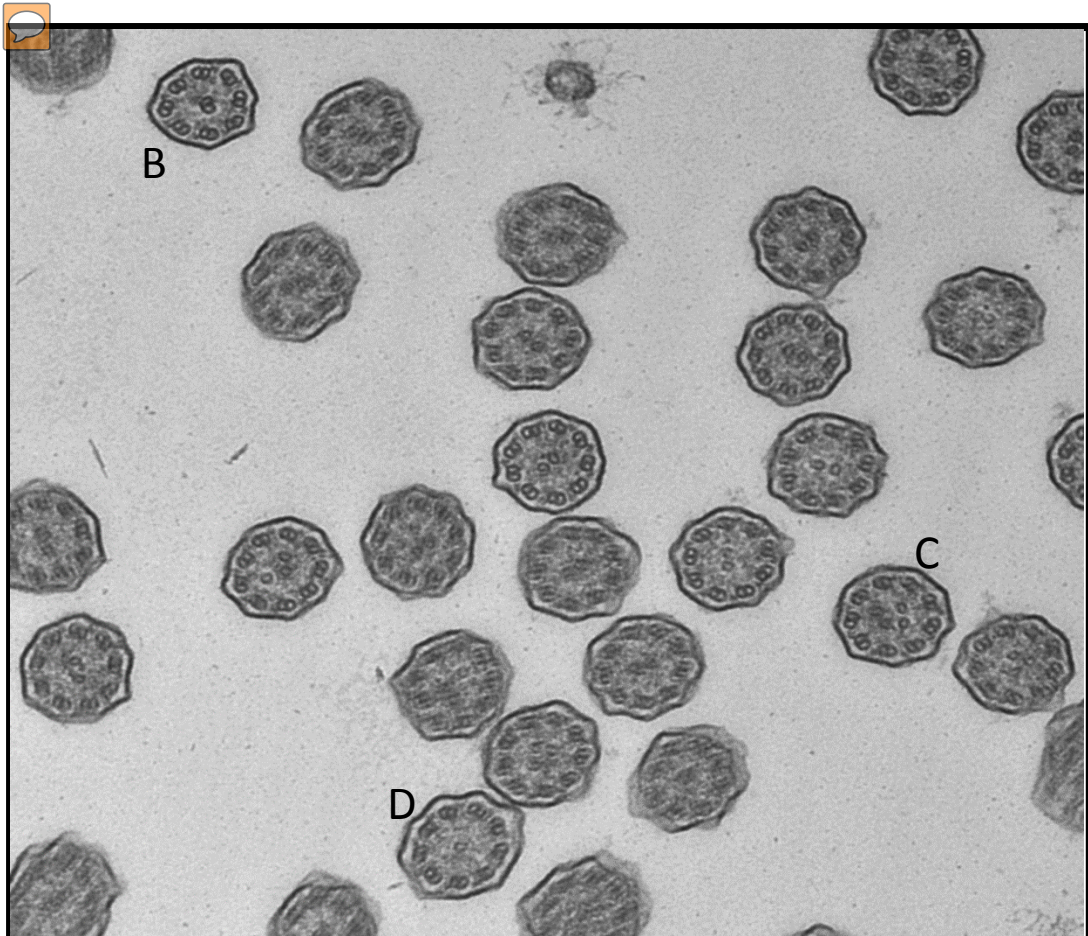


Outer and inner  
dynein arm defect

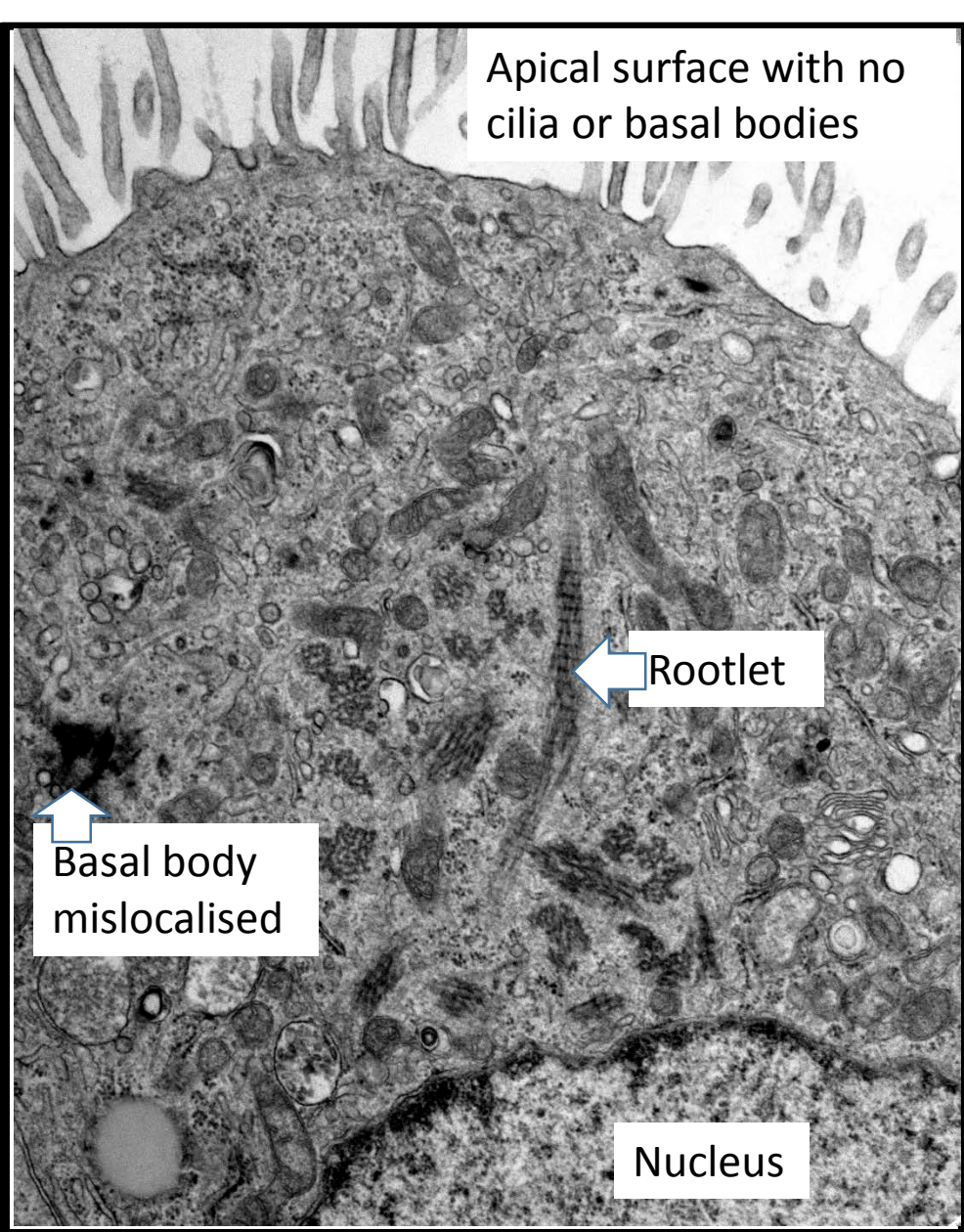


Microtubular  
disorganisation and inner  
dynein arm defect





Central complex defect



Mislocalisation of basal bodies with few or no cilia