New insights in Primary Ciliary Dyskinesia

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ABSTRACT

Introduction: Primary ciliary dyskinesia (PCD) is a rare genetic disease with an estimated prevalence of 1:20.000 births. It is characterized by abnormal motility of cilia, leading to impaired mucociliary clearance, and subsequent infection and chronic inflammation of the airways. PCD also affects spermatozoa and cilia in the Fallopian tubes, contributing to fertility issues; dyskinesia of embryonic nodal cilia causes a random distribution of the organs.

Areas covered: An overview of the history, genetics, clinical manifestations in children and adults, diagnostic tests, treatments, and prognosis are reviewed. We also discuss current research and future prospects of PCD.

Expert opinion: As PCD comprises defects in all organs with motile cilia, patients have a variety of clinical manifestations, often characterized by their presence from birth. Because of the non-specific symptoms, PCD is often confused with other diseases such as cystic fibrosis. There is no gold standard diagnostic test and a variety of diagnostic tests are required, including high-speed video analysis and transmission electron microscopy. Reanalysis following primary cultures of the epithelial cells can help to differentiate primary from secondary defects. Despite being a genetic disease, due to the genetic heterogeneity of PCD, gene analysis can currently only explain 65% of the cases. There is no treatment for PCD, and therapeutic options that contribute to the wellbeing of the patients are based on expert opinion.

KEYWORDS

Clinical features, diagnostic methods, genetics, Primary Ciliary Dyskinesia, therapy

ARTICLE HIGHLIGHTS

- Primary Ciliary Dyskinesia (PCD) is a rare genetic disease characterized by abnormal mobility of motile cilia and sperm.
- The manifestations of PCD are characterized by their presence from birth, their persistent clinical course throughout life.
- The main clinical manifestations chronic infections in upper and lower airways, due to the inefficient mucociliary transport.
- The diagnosis of PCD lacks a "gold standard" and a combination of tests is therefore needed.
- Despite the existence of therapeutic options that contribute to wellbeing of patients, to date no specific treatment exists for PCD.

LIST OF ABBREVIATIONS

PCD- Primary Ciliary Dyskinesia

CF- Cystic Fibrosis

COPD- Chronic Obstructive Pulmonary Disease

TEM- Transmission Electron Microscopy

CBP- Ciliary Beat Pattern

CBF- Ciliary Beat Frequency

HSVM- High Speed Video Microscopy

nNO- exhaled nasal Nitric Oxide

MCT- Mucociliary Transport

SCD- Secondary Ciliary Dyskinesia

PICADAR- the PrImary CiliAry DyskinesiA Rule

ALI- Air-Liquid Interface

ODA- Outer Dynein arms

IDA- Inner Dynein arms

HRCT- High-Resolution Computed Tomography

ODA-DC- ODA docking complex system

LCI- lung clearance index

QOL- quality of life

GCMCC- Genetic Disorders of Mucociliary Clearance Consortium

ERS- European Respiratory Society

1. Introduction

Primary ciliary dyskinesia (PCD) is a rare genetic disease characterized by abnormal mobility of motile cilia. As a consequence, clearance of secretions, bacteria, pollutants and allergens is impaired from the upper and lower airways. PCD is the second most common congenital condition of the respiratory airways after cystic fibrosis (CF), with an estimated prevalence of 1/20.000 births (ORPHA244).

The main clinical manifestations are chronic symptoms and infections in upper and lower airways, due to the impaired mucociliary transport. Symptoms often start neonatally. During the infancy and childhood, the manifestations are daily wet cough, persistent rhinitis and serous otitis. Bronchiectasis can present in infancy, and by adulthood is almost universal[1]. PCD also affects spermatozoa and cilia in the Fallopian tubes, contributing to fertility issues. Moreover, dyskinesia of embryonic nodal cilia causes a random distribution of the organs, which produces *situs inversus* in 50% of the cases and situs ambiguous in 6%[2]. The inheritance of PCD is autosomal recessive in the majority of the cases. In terms of genetics, PCD is a heterogeneous disease and to date mutations in 39 genes have been identified, accounting for 65% of PCD cases[3, 4].

Diagnosis of PCD is based on identifying the clinical manifestations, nasal Nitric Oxide (nNO) levels, analysis of the ciliary ultrastructure by Transmission Electron Microscopy (TEM), cilia function analysis by High Speed Video Microscopy (HSVM), and genetic testing [5]. However, the heterogeneity of the disease and the similarities with other chronic respiratory diseases often cause delay in the diagnosis.

To date no specific treatment exists for PCD. However, therapeutic options that contribute to wellbeing include regular monitoring of respiratory and auditory function, regular airway clearance of secretions through physiotherapy and physical exercise, and the use of antibiotics for treating airways infections[1].

2. Historical Background.

The association of *situs inversus* and bronchiectasis was first described by Siewert in 1901 and published shortly after in 1904 [6]. In 1933, Kartagener described a group of patients who presented with bronchiectasis, sinusitis and *situs inversus*[7] leading to the term"Kartagener's syndrome".

In 1975, Afzelius identified ultrastructural abnormalities in the spermatozoa of a group of infertile men who had also chronic bronchiectasis[8]. Subsequent studies showed that these patients had similar ultrastructural alterations in their respiratory cilia[9]. Moreover, in 1977 Elliasson described a group of patients presenting with similar symptoms and ultrastructural abnormalities, but no *situs inversus*[10] . Therefore, since 1980 the term "immotile cilia syndrome" was coined, reserving "Kartagener syndrome" to define the triad of sinusitis, bronchiectasis and *situs inversus*[11, 12].

In 1988, Rossman and Newhouse demonstrated that symptoms may be due not only to the total immobility of cilia, but also to dyskinetic and inefficient movement. Therefore, the term PCD was proposed instead of "immotile cilia syndrome"[13].

During late 80´s it was shown that mucociliary transport could be altered not only by inherited ultrastructural defects of cilia, but also by factors such as recurrent viral or bacterial infections, resulting in ciliary dysfunction that could reproduce the clinical picture of the PCD[14].The name "secondary ciliary dyskinesia (SCD)" was used to refer to the transient alteration of mucociliary transport, as opposed to PCD which refers to its hereditary and permanent character [15].

Recent advances in HSVM and in genetics have further opened our understanding of disease, such that the term 'primary ciliary dyskinesia' is probably inadequate to describe all variants, for example, some patients have reduced numbers of motile cilia.

3. Clinical manifestations in children.

The manifestations of PCD are characterized by their presence from birth, their persistent and progressive clinical course throughout life, simultaneously affecting all organs in which the motile cilia exert their function (Table 1).

Diagnosis of PCD is frequently delayed [16, 17], in part because patients present with symptoms (rhinitis, secretory otitis media, cough and recurrent bronchitis) that are common in the non-PCD population. A recent systematic review aimed to investigate how symptoms of PCD vary by age, but reported that most studies were small, with poor stratification by age [18].

Approximately 40–55% of affected patients have *situs inversus* and 6-12% have heterotaxy (*situs ambiguus*), which can be associated with cardiac defects[19, 20]. Mild fetal cerebral ventriculomegaly could be an early sign of PCD[21], perhaps related to ependymal ciliary dysfunction, and hydrocephalus is a very rarely reported [18].

PCD usually presents with unexplained neonatal respiratory distress (75-85%)[22]. Symptoms start after a few hours of life and often need prolonged supplemental oxygen[23]. Lobar collapse of the upper and middle lobe is a frequent complication in the neonatal period. This is in contrast to older children and adults with PCD where radiological findings typically involve the middle and lower lobes.

Chronic nasal congestion and daily wet cough are common features throughout life [18, 24]. Symptoms might partially improve with antibiotic therapy or changes of season, but do not resolve completely [23, 25].

Chronic or recurrent otitis media with effusion is very common and is often associated with conductive hearing difficulty. Persistent otorrhoea can occur after ventilation tube insertion. Symptoms of sinusitis often develop in childhood. However, nasal polyposis is rare in children [1, 26].

4. Clinical manifestations in adults.

Daily wet cough and chronic rhinorrhea persist into adulthood. Rhinitis responds poorly to standard treatments and is often complicated by sinusitis in older children and adults. Nasal polyposis is said to be less common than in CF. Ear problems generally improve with age, but patients may have permanent conductive hearing loss [27].

PCD patients often have hypoplasia of the paranasal sinuses, and especially, frontal sinus aplasia (Figures 1B and 1C: Radiological typical findings in an adult patient with PCD), evidenced when performing a computed tomography scan (CT).

Although bronchiectasis can appear in infancy, it is almost universal by adulthood. Cylindrical or saccular bronchiectasis involves the middle and lower lobes (Figure 1A) and the lingula. In addition, patients typically have radiological findings of peribronchial thickening, atelectasis and air-trapping [28, 29].

Patients often have poor pulmonary function, ranging from mild to severe as a consequence of recurrent and chronic infection [30, 31].

Fertility problems are apparent in some adults. Some men have alive but immotile sperm, making them infertile. Impaired ciliary function in the fallopian tubes delays ovum transport which might cause infertility. However some patients are fertile [32], and good studies are needed to understand the true prevalence of infertility[33]. There have been very rare reports of ectopic pregnancy in patients with PCD but it is not clear if it is more common than in the general population[34, 35].

5. Diagnosis and treatment of PCD.

5.1 General considerations

The diagnosis of PCD lacks a “gold standard” and a combination of tests is therefore needed to get a conclusive diagnostic. A European Respiratory Society Task Force has recently published evidence-based guidelines for making or excluding the diagnosis [36].

The first step is identifying patients with a compatible clinical history for diagnostic testing. Two studies propose screening tools based on symptom scores [24, 37]. PICADAR was developed using symptom data from >600 patients referred for diagnostic testing [24]. A regression model identified symptoms that were predictive of PCD for inclusion in the 7-item clinical tool (27). Another study developed a predictive score based on 4 clinical symptoms, demonstrating that by using 'enhanced questions' (e.g. "early onset year-round wet cough" rather than simply "wet cough") the specificity improved, but sensitivity reduced [37].

Clinical manifestations of PCD are similar to other diseases such as CF, immunodeficiency, *Aspergillus* or *Tuberculosis* infections, chronic gastro-esophageal reflux with aspiration, and alpha-1 antitrypsin deficiency[38]. Some of these conditions have conclusive diagnostic tests that could be considered before starting with PCD diagnostic tests.

5.2 Diagnostic tests

5.2.1 nNO levels

nNO is the recommended initial test in symptomatic patients[36, 40] and low levels of nNO should prompt referral for further tests. Despite having a good sensitivity, a small number of PCD patients have normal nNO levels so full diagnostic testing should not be prevented if the clinical suspicion is high[36], and low nNO levels are also associated with other similar pathologies as CF. Although it can contribute to the final diagnostic decision, nNO cannot be used as a conclusive tool because of false positive and false negative cases[5]. Moreover, it cannot be applied to children <6 years old.

5.2.2 Study of ciliary beat pattern and ciliary beat frequency

Study of ciliary mobility by HSVM has good sensitivity and specificity [36, 41]. Analysis should be conducted by experienced personnel and requires sophisticated equipment. Differentiating primary and secondary causes of PCD can be extremely difficult; repeating

the HSVM following culture at an air-liquid interface (ALI) cell cultures, can help with the distinction [36, 42].

5.2.3 Genetic tests

Approximately 65% of patients have mutations in one of the 39 known PCD genes. Current guidelines suggest that genetics testing is directed by other diagnostic tests [36] but as knowledge of genetic mutations improves, genotype is likely to take a more prominent role in diagnostic algorithms. There are genotypes which are difficult to diagnose by other tests where genetics testing already has an important role e.g. mutations in CCNO and RSPH4 (See section PCD genetics).

5.2.4 Transmission Electronic Microscopy

TEM allows study of the ciliary axoneme. The most common ultrastructure defects that cause ciliary movement abnormalities are: outer dynein arm (ODA) -defects (25-50%), combined ODA and inner dynein arms (IDA) deffects (25-50%), IDA defects associated with microtubular disorganization (15%) and central microtubule pair defects (5-15%)[36, 43, 44]. The pathogenicity of isolated IDA is controversial. Central pair defects are characterized by a mix of both normal and abnormal cilia necessitating protocols that count a large number of cilia in cross section (e.g. >100) to ensure that false negatives are avoided.[45]. Moreover, some defects cannot be detected by classical TEM in 15-20% of PCD patients [36]. Despite limitations with TEM, this test is excellent for confirming PCD in patients with 'hallmark' ultrastructural defects (Figures 2A, 2B and 2C) [36].

5.2.5 Differentiated airway epithelial cells cultures

Chemical and biological agents (e.g. viruses and bacteria) can alter ciliary activity, disturbing the mucus clearance and leading to pulmonary damage. It can be difficult to discriminate SCD from PCD by HSVM, making diagnosis difficult if TEM and genetics tests are normal. In these cases, it is informative to culture the nasal epithelial cells obtained from the initial

brushing using an ALI technique [42] repeating the HSVM in those re-differentiated cells: if ciliary dyskinesia is secondary, the ciliary activity of re-differentiated cells will be normal, but if it is due to PCD, ciliary dyskinesia will persist in the cultured cells [36, 42].

5.2.6 Immunofluorescence analysis of cilia proteins

Immunofluorescence staining of specific ciliary structure proteins is a potential diagnostic test. However, to date there have been no studies investigating its role as a diagnostic test. [46]

5.3 Treatment of PCD

Currently, there is no specific treatment to restore ciliary mobility. Therefore, management is usually based on evidence from other more common disorders e.g. CF and rhinosinusitis.

Firstly, it is necessary to monitor airways disease, upper airways symptoms and audiology. Consensus guidelines recommend that treatment is based in combinations of antibiotics and airway clearance therapies[46, 47]. Airway clearance should be improved by regular chest physiotherapy and physical exercise, anecdotally providing important benefits for patients[48]. It is important to treat infections tailoring medication to sputum cultures. An international trial is currently investigating the role of azithromycin to prevent exacerbations.[49] To treat *Pseudomonas* colonization, management is usually extrapolated from CF. Whereas mild exacerbations are often treated with oral antibiotics and physiotherapy, severe or refractory exacerbations require intravenous antibiotics[50].

The role of agents such as hypertonic saline or mannitol, recombinant deoxyribonuclease, N-acetylcisteine or anti-inflammatory treatments remain unclear in PCD patients[48, 51]. Bronchodilators and inhaled corticosteroids should be considered in patients with PCD who also have co-existing asthma or bronchial reversibility, but are not indicated routinely[48].

Infection prevention is recommended in PCD patients, so routine vaccines, as well as all PCD patients should receive routine immunizations plus *Pneumococcal* and *Influenza* vaccinations [48, 52, 53].

Chronic rhinorrhea might be helped by sinus rinses. Rhinosinusal superinfections are treated with antibiotics. Surgical treatment of rhinosinusitis is controversial. Treatment of secretory otitis media by timpanostomy is also controversial particularly as it may be complicated by prolonged postoperative chronic otorrhea, therefore conservative management is usually advocated. The periodic use of hearing aids is often recommended by experts [54, 55].

In severe cases of PCD with end stage lung disease, lung transplantation may be an option[56]. Surgical resection remains controversial[57]. Cardiac defects are 200-fold higher in PCD than in general population[19]. The spectrum of cardiac defects is wide and management depends on the lesion[58]. Careful pre-operative planning by a multidisciplinary team of cardiologists, pulmonologists and intensivists is needed, since the post-operative course is often complicated.

Genetic counselling and management should be offered to patients that want to have children, considering that is an inherited disease. Intracytoplasmic spermatozoa injections could facilitate conception in males with immotile sperm[33]. In addition, in vitro fertilization and intra-uterine implantation could help female patients with PCD with decreased fertility[59].

6. Evolution and prognosis.

The literature regarding longitudinal lung function in adult and pediatric PCD is conflicting; studies show stability in lung function over time, wide variability or steady deterioration. Some reports [60-62] have indicated that lung function can be stabilized after diagnosis and initiated treatment, even in patients with late diagnosis and poor lung function. However, a study of 74 PCD patients showed a great variation in the course of lung function following

diagnosis [30] with pulmonary function remaining stable in >50%, deteriorating in one third, and improving in others. Pulmonary function was already impaired at the first spirometric measurement in more than one-third of children with preschool PCD diagnosis demonstrating that early diagnosis does not always protect against impaired lung function, and that PCD is a disease of serious threat to lung function even at preschool age.

Despite widespread use, there is no evidence that lung function alone is useful in the assessment of progression of lung disease in PCD. Imaging techniques like high-resolution computed tomography (HRCT) can be more sensitive. In one study spirometry was less accurate than HRCT in detecting changes associated with progression of lung disease; structural damage worsened despite stable spirometry, and longitudinal evolution of FEV1 was not significantly associated to the change in HRCT total score[63]. In a recent pediatric cohort study, moderate lung function worsening, progressive structural lung impairment detected by HRCT and a significant correlation between HRCT score and FEV1 decline were reported [64]. Adult PCD studies have also shown that HRCT severity scores are associated with disease progression with a very high frequency of bronchiectasis (96-100%)[28, 65].

There is little information about the evolution of PCD into adult life. Even data on mortality is extremely limited. In a large British clinic, the median age at death was 65 years (range 31-75 years) and no patients underwent lung transplant [65]. In an American cohort 38% of adults experienced respiratory failure and had an FEV1 ≤ 40% with some patients having or being considered for lung transplantation[31]. Bilateral lung transplantation for end-stage PCD is probably rare in Europe.

There is not much knowledge about features associated with disease progression in PCD. Isolation of *Pseudomonas aeruginosa* has been associated with increased severity of disease and mortality in CF and in adult non-CF bronchiectasis. In PCD *P. aeruginosa* colonization seems to be a marker of disease severity reflecting cases with more impaired FEV1 and

higher radiological extent of bronchiectasis, but it is not associated with longitudinal decline of FEV1[65].

Ciliary ultrastructure and the type of genetic mutations could be related to prognosis Microtubular defects (CCDC39, CCDC40) and CCNO mutations have been suggested to be associated with a more severe phenotype. This needs confirming through large collaborative studies.[23, 65]

7. Genetics of PCD.

PCD is a Mendelian autosomal recessive and genetically heterogeneous disorder (with the exception of two rare X-linked syndromic genes, RPGR (MIM 312610) and OFD1 (MIM 300170), which combine PCD with retinitis pigmentosa and orofacial digital syndrome, respectively, and PIH1D3 (MIM 300933), an X-linked gene causing non-syndromic PCD. For autosomal recessive causes, pathogenic mutations must be find on bi-allelic genes. No documented cases of heterozygous mutations in two different PCD genes have been associated with human PCD.

There are currently 39 known genes associated with PCD (Table 2), with new genes being discovered at a rapid pace. These gene-mutations account for only 60-70% of individuals affected with PCD. Of the 39 known genes, 10 encode proteins of the ODAs or the ODA docking complex system (ODA-DC) (DNAH5 [MIM 603335; 15–21% prevalence], DNAH11 [MIM 603339; 6% prevalence], CCDC114 [MIM 615038], TTC25 [MIM 617095], DNAL1 [MIM 610062], DNAI1 [MIM 604366], DNAI2 [MIM 605483], NME8 [MIM 607421], ARMC4 [MIM 615408] and CCDC151 [MIM 615956]) mutations of which generally cause isolated outer dynein arm deficiency. PCD mutations in genes controlling ODA complex protein generally are translated in immotile cilia phenotype, since ODA controls effective movement of cilia. Eleven genes encode cytoplasmic proteins involved in assembly and transport of the dynein arms into axonemes (SPAG1 [MIM 603395], DNAAF1

[MIM 613190], DNAAF2 [MIM 612517], DNAAF3 [MIM 614566], HEATR2 [MIM 614864], DYX1C1 [MIM 608706], ZMYND10 [MIM 607070], DNAH1 [MIM 603332], LRRC6 [MIM 614930], CCDC103 [MIM 614677] and C21orf59 [MIM 615494]) mutations of which combine ODA and IDA deficiency.

Thirteen genes with PCD causal mutations are associated factors of the N-DRCs, including CP, RS, CP and MT (N-DRC regulators CCDC39 [MIM 613798], CCDC40 [MIM 613799], CCDC65 [MIM 611088], CCDC164] [MIM 615288]) and GAS8 [616726]; RS regulators (RSPH1 [MIM 609314], RSPH4A [MIM 612647], RSPH3 [MIN 616481], DNAJB13 [MIM 610263] and RSPH9 [MIM 612648]); CP microtubules (HYDIN [MIM 610812]; regulators of multi-ciliated cell differentiation and ciliogenesis CCNO [MIN 615872] and MCIDAS, which loss results in a reduction in the number of motile cilia) which cause defects in cilia movements but not immotile cilia. More recent studies added new gene defects that affect to energy regulatory proteins of cilia such as adenylate kinase 7 (AK7) mutation in PCD subjects[66].

Gene therapy is an area of interest. In a proof of principal study gene editing using transcription activator-like effector nucleases (TALENs) corrected a DNAh11 mutation in an *ex vivo* model[67]. Other promising gene edition repair strategies include clustered regularly interspaced short palindromic repeats (CRISPRs). However, delivery to cells in humans, off-target effects and unintended DNA cleavage, inter- and intra-chromosomal rearrangements and variable DNA cleavage efficiencies may limit the rapid progression of PCD gene repair.

8. On-going research and future prospects.

Basic Science: Since Afzelius and Pedersen proposed the unifying role of cilia to explain the syndrome in 1976, basic scientists have developed our understanding of the underlying pathophysiology and genetics of PCD. The first PCD-causing gene was described in 1999 [68] and there are now over thirty known genes. As previously discussed, personalized

treatments targeting specific genetic defects, have become a recent area of interest. Further advances to characterize and correct mutations or protein function will require pre-clinical models including human *ex vivo* and animal *in vivo* models. Animal models have been pivotal to our understanding of the diverse aspects of biology in motile cilia; these include molecular composition, mode of assembly, motility mechanisms, PCD pathogenesis and PCD candidate gene identification. Cilia and flagella have been conserved through much of evolutionary history, and models which have been used to develop our understanding of PCD include invertebrate systems such as *Chlamydomonas reinhardtii* [69-71]and vertebrate models such as *Xenopus* [72, 73] and zebra fish[74-76]. Genetic PCD mice are the only models with lungs, and while displaying aspects of human PCD such as rhinitis and laterality defects, they do not develop significant chest disease despite immotile respiratory cilia; importantly most but not all genetic backgrounds have the potential to develop severe morbidity from hydrocephalus with differences in expressivity and/ or penetrance dependent on the background strain [77-80]. The power of these various models in cilia research has been explored in a number of outstanding review manuscripts[79, 81-83].

Epidemiology: Most epidemiological research on PCD come from small datasets. A recent systematic search found 50 publications with clinical information[18] and reported that data quality was often poor, results were not age-stratified and populations were highly selected from specialist clinics. Only a few studies have described longitudinal data, such as changes of lung function[84, 85] or growth [85] over time. Collaborations between multiple centers are starting to provide into insights to gene-phenotype associations, with a suggestion that CCDC39/ CCDC40 are associated with more severe lung disease[23].

Diagnostic testing: There are considerable differences between centers and countries with respect to diagnostic testing, [45, 86, 87] which is not surprising given the lack of a 'gold standard'. A number of studies have investigated the accuracy of established tests to diagnose

PCD including HSVM [41, 88], TEM [41, 89-93], reanalysis following cell culture [42, 94] and genetics testing [23, 95]. Major difficulties for interpreting these accuracy studies include the lack of a reference standard, and no global standardization of methods. Explorations to improve diagnostic accuracy in 'difficult cases', where TEM is normal and pathogenic mutations have not been identified, have included electron tomography [96, 97] and radioaerosol mucocilary clearance [98, 99]. Electron tomography is an advanced EM technique used for the visualisation of structures in 3D. Resolution of approximately 5nm and the 3D information have been used to provide important insights into the structure of molecules and their interactions within the axoneme of *Chlamydomonas reinhardtii,* and more recently to confirm the absence of Hydin or DNAh11 molecules as causes of PCD[100, 101]. This has led to an interest in the potential of electron tomography as a diagnostic tool in patients with apparently normal ultrastructure by standard TEM[100].

Treatment and outcome measures: To date there have been no powered randomized trials to guide choice of treatments, therefore clinical management varies widely [87]. An international trial is currently investigating the role of azithromycin to reduce pulmonary exacerbations [49], and a further study is investigating the effect of sodium channel inhibition on lung function (https://clinicaltrials.gov/ct2/show/NCT02871778). One of the obstacles to clinical trials has been lack of reliable and responsive outcome measures. Spirometry has the disadvantage of declining slowly in most PCD patients[84, 102], it cannot be measured in young children and infants, and it is insensitive to structural disease evident on high resolution CT-imaging[103]. High resolution CT is sensitive to lung damage but is not suitable for repeated measures because of the radiation exposure. In recent years there has been renewed interest in lung clearance index (LCI) measured by multiple-breath washout in CF, and several studies have now been conducted in PCD. LCI is more sensitive than spirometry in PCD [104-107] and can be used in infants and young children. We need

longitudinal data, and standardized methods before LCI can be secured as an outcome measure for PCD clinical trials[107, 108].

Patient reported outcome measures are advocated for clinical trials to assess the impact disease has on the patient's daily functioning; the QOL-PCD are age-appropriate health-related quality of life measures. These tools were developed in the English language to be linguistically and culturally equivalent in North America and Europe[109, 110]. Validation studies have confirmed reliability of QOL-PCD, and the instruments have been translated into a number of languages[111]. This international approach is important for rare diseases since multi-national clinical trials will enable recruitment of sufficient patients.

Research collaborations: Over recent years, advances in the field of PCD have occurred through separate collaborations of clinicians and of scientists. Several international initiatives have stimulated these advances including the North American Genetic Disorders of Mucociliary Clearance Consortium (GDMCC)[23, 112-114], Ciliopathy Alliance, Gordon Conferences, two network European Respiratory Society (ERS) Task Forces[16, 36, 87, 115, 116] and European FP7-funded BESTCILIA [49, 109, 117]. To maintain the momentum of these collaborations and to build on their successes, there was a need for networks to bring clinicians and scientists together. BEAT-PCD (http://www.beatpcd.org/) is a collaboration supported by EU- Framework Horizon 2020 (COST Action BM1407). The global network includes experts from diverse clinical specialties (e.g. pediatric & adult pulmonology, ENT, physiotherapy) and multidisciplinary scientists (e.g. genetics, imaging, cell biology, microbiology, and bioinformatics). They aim to identify gaps in knowledge, and then facilitate PCD-related research to identify mechanisms, study disease patterns and progression, define outcome measures, improve clinical management and identify high priority therapies. In the United States, the PCD Foundation (http://www.pcdfoundation.org/)

is promoting the advance of PCD through annual conferences, community education, patient advocacy, and research funding.

In summary, PCD research is gathering momentum through successful collaborations, but many questions remain unanswered in this exiting research arena.

9. Conclusion.

In conclusion, PCD is a congenital disorder that affects upper and lower airways. Clinical manifestations can vary depending on the patient and their age. As a consequence of the variety of manifestations and the similarities between PCD and other chronic respiratory diseases, patients fail to be referred for diagnostic testing. There is no gold standard diagnostic test, and a combination of nNO levels, analysis of cilia ultrastructure by EM; analysis of ciliary function by HSVM, and genetic testing can be used [36]. Because there are a large number of implicated genes and mutations, and because many genes have yet to be found, genetic testing is far from being a definitive diagnostic method for PCD. Moreover, with a lack of specific treatments, management is limited to careful monitoring, physiotherapy and physical exercise, as well as the use of antibiotics to treat the infections. The lack of a definitive diagnostic test and absence of specific treatments highlight the relevance of research in this area. Expert multidisciplinary reference centres working together through international networks are promoting advances in diagnostic and clinical management (http://ern-lung.org/ and GDMCC).

10. Expert opinion

Cilia disorders are known as ciliopathies. Ciliopathies of the primary cilia are responsible for diverse sensorial syndromes and deficits, such as disorders of smell (anosmia), hearing (sensorineural hearing loss), and vision (retinal degeneration). Primary ciliopathies are also responsible for conditions such as polycystic kidney, polydactyly, central nervous system malformations, developmental delay, heart, gonadal and craniofacial malformations, as well

as basal carcinomas. These features can occur in various combinations in a patient, leading to syndromes such as Usher (hearing and vision loss), Bardet Biel, Alstrom, Joubert, Merckel Gruber, Senior Locken syndrome, and probably others that still unknown. PCD is a ciliopathy caused by abnormal motile (rather than primary) cilia. Association of motile and primary ciliopathies in the same patient are unusual.

PCD includes a heterogeneous group of genetic disorders. This heterogeneity is related with a variety of phenotypes, including different abnormal patterns of ciliary structure and function. Consequently, diagnostic testing can be a challenge. A definitive diagnosis is essential, mainly for the patient's care, but also for recruitment to research studies.

Children with chronic respiratory infections that appear from birth, simultaneously affecting upper and lower airways, with little or no improvement at any time of the year are highly suspicious of PCD. Newborns with no identifiable cause of neonatal respiratory disease, with or without organic laterality disorder are highly suspicious of PCD if symptoms persist. It is important that physicians are aware of the symptoms of PCD, and that they refer appropriate patients for diagnostic testing.

To diagnose PCD patients, having a strong clinical history (including nNO levels if it is possible), the starting point is HSVM analysis of the ciliary function, followed by TEM analysis of the ciliary structure. In case of poor health of the initial sample, ALI primary cultures of nasal or bronchial epithelial cells can be done, and ciliary function and structure re-analysed in order to differentiate primary from secondary defects. Sometimes it is necessary to repeat the sampling and tests, especially the motility studies. These tests are complex and it is essential that the studies are carried out by experts.

The complexity of the diagnostic tests has led to development of reference centers, with specialist in PCD professionals. These specialist centers are expensive and require funding. The difficulties caused by lack of a gold standard test, the necessity of specialists to carry out

the diagnostic tests, and the funding these services require has led to differences in provision between countries.

Clinical management of these patients requires lifetime medical care. The treatment should be adapted to the clinical evolution, whereas respiratory physiotherapy is needed daily. Treatment with antibiotics is often needed, and should be adapted according to the patient's clinical status and microbiological studies. Otitis media with effusion is common in patients with PCD, leading to recurrent acute otitis media, hearing loss, and potentially chronic suppurative otitis media even with cholesteatoma. Therefore, in some cases surgical treatment is necessary. In children with hearing loss, hearing aids should be considered to prevent speech delay and educational compromise.

In terms of new therapies, there are currently clinical trials with inhaled solution of epithelial sodium channel inhibitors in subjects with PCD investigating the effect on pulmonary function. Because of the genetic heterogeneity that characterises PCD, gene therapy is more difficult to achieve than in monogenic diseases. However, gene therapy focused in the most prevalent and better described mutations could be a reality providing a definitive curation of patients with specific mutations.

In spite of what we have learnt to date, basic and clinical aspects of the disease remain poorly understood. The prevalence and severity of clinical symptoms and how these vary with age, sex, and country are not known. there is poor data about progression of disease over time, mortality and evolution of lung function. Moreover, there is a lack of prognostic factors, which could be useful for the clinical management of PCD patients. Finally, genotyping can only explain around 65% of PCD cases, so further research is needed to detect further causative genes.

PCD genes	Prevalence in PCD	Protein localization/ function	Ciliary ultrastructure defect	Phenotypic presentation	Ref.
DNAH5	++++	ODA	ODA defect	Immotile cilia	[118-120]
DNAI1	+++	ODA	ODA defect	Immotile cili	[121-125]
DNAI2	++	ODA	ODA defect	Immotile cilia	[126]
DNAL1	+	ODA	ODA defect	Impaired motility	[127]
CCDC114	++	ODA docking complex	ODA defect	Immotile cilia	[128, 129]
TTC25	+	ODA docking complex	ODA defect	Immotile cilia	[130]
CCDC151	++	ODA targeting and docking	ODA defect	Immotile cilia	[131, 132]
ARMC4	++	ODA docking complex	ODA defect	Immotile cilia	[133, 134]
CCDC103	++	Cytoplasmic, ODA assembling	ODA ± defect	Immotile cilia	[135]
NME8 (TXNDC3)	+	ODA	Partial ODA defect	Immotile cilia	[136]
DNAAF1 (LRRC50)	++	Cytoplasmic, DA assembling	ODA and IDA defects	Immotile cilia	[137, 138]
DNAAF2	++	Cytoplasmic,	ODA and IDA	Immotile cilia	[139]

		DA assembling	defects		
DNAAF3	+	Cytoplasmic, DA assembling	ODA and IDA defects	Immotile cilia	[140]
LRRC6	++	Cytoplasmic, DA assembling	ODA and IDA defects	Immotile cilia	[141, 142]
HEATR2	+	Cytoplasmic, DA assembling	ODA and IDA defects	Immotile cilia	[143]
DYX1C1	+	Cytoplasmic, DA assembling	ODA and IDA defects	Immotile cilia	[144]
C21orf59	+	Cytoplasmic, DA assembling	ODA and IDA defects		[74]
ZMYND10	++	Cytoplasmic, DA assembling	ODA and IDA defects	Immotile cilia	[73, 145]
SPAG1	++	Cytoplasmic, DA assembling	ODA and IDA defects	Immotile cilia	[146]
DNAH1	+	IDA	IDA defects	Reduced cilia motility	[147]
RPGR	+	Cytoplasmic	Normal	Syndromic, PCD with retinitis pigmentosa	[148, 149]
OFD1	+	Cytoplasmic	Normal	Syndromic, PCD with orofacial digital syndrome	[150]

PIHID3	+	Cytoplasmic	ODA and IDA defects	Mostly immotile	[151]
DNAH11	+++	ODA	Normal	Hyperkinetic cilia, reduced amplitude	[152, 153]
HYDIN	+	Central pair C2b defect with normal body composition	Normal	Reduced amplitude, discoordination	[154]
CCDC164 (DRC1)	+	Nexin-dynein regulatory complex defect	Mostly normal/with DRC defects	Reduced bending amplitude	[70]
CCDC65 (DRC2)	+	Nexin-dynein regulatory complex defect	Mostly normal	Impaired motility	[74]
GAS8	+	Nexin-dynein regulatory complex defect	Mostly normal	Reduced bending amplitude	[155]
CCDC39	+++	Nexin-dynein regulatory complex defect and IDA assembling	MT disorganisation and IDA defect	Hyperkinetic, stiff cilia	[156, 157]
CCDC40	+++	Nexin-dynein regulatory	MT disorganisation	Hyperkinetic, stiff cilia	[156, 158]

		complex defect and IDA assembling	and IDA defect		
RSPH9	+	Radial spoke	MT disorganisation (CP-RS defect)	Circular beat	[159-161]
RSPH4A	++	Radial spoke	MT disorganisation (CP-RS defect)	Circular beat or stiff cilia	[159-161]
RSPH1	++	Radial spoke	MT disorganisation (CP-RS defect)	Different beating patterns	[160, 162, 163]
RSPH3	+	Radial spoke	MT disorganisation (CP-RS defect)	Reduced bending amplitude	[164]
DNAJB13	+	Radial spoke/cytoplasm	Abnormal percentage of cilia lacking central microtubules	Inmotile	[165]
CCNO	+	Apical cytoplasm	Oligocilia (residual axoneme normal)	Reduction of multiple motile cilia	[166]
MCIDAS	+	Apical	Oligocilia	Reduction of	[72]

		cytoplasm	(residual axoneme normal)	multiple motile cilia	
DNAH8	+	Not available			[167]
AK7	+	Cytoplasm	Normal	Reduced ciliary beat frequency	[66]

Table 2: +, genetic mutations causing <1% of all PCD; ++, genetic mutations causing 1–4% of all PCD; +++, genetic mutations causing 4–10% of all PCD; ++++, genetic mutations causing >15% of all PCD; CP-RS, central pair- radial spoke; DA, dynein arm; DRC, dynein regulatory complex; IDA, inner dynein arm; IDA + MT, inner dynein arm defect with microtubule disorganization; NGS, next-generation sequencing panels commercially available; PCD, primary ciliary dyskinesia; ODA, outer dynein arm; Oligocilia, the presence of only few cilia.

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** *In this article it can be ascertained the lack of awareness of the physicians of this disease, as well as the patients themselves.*

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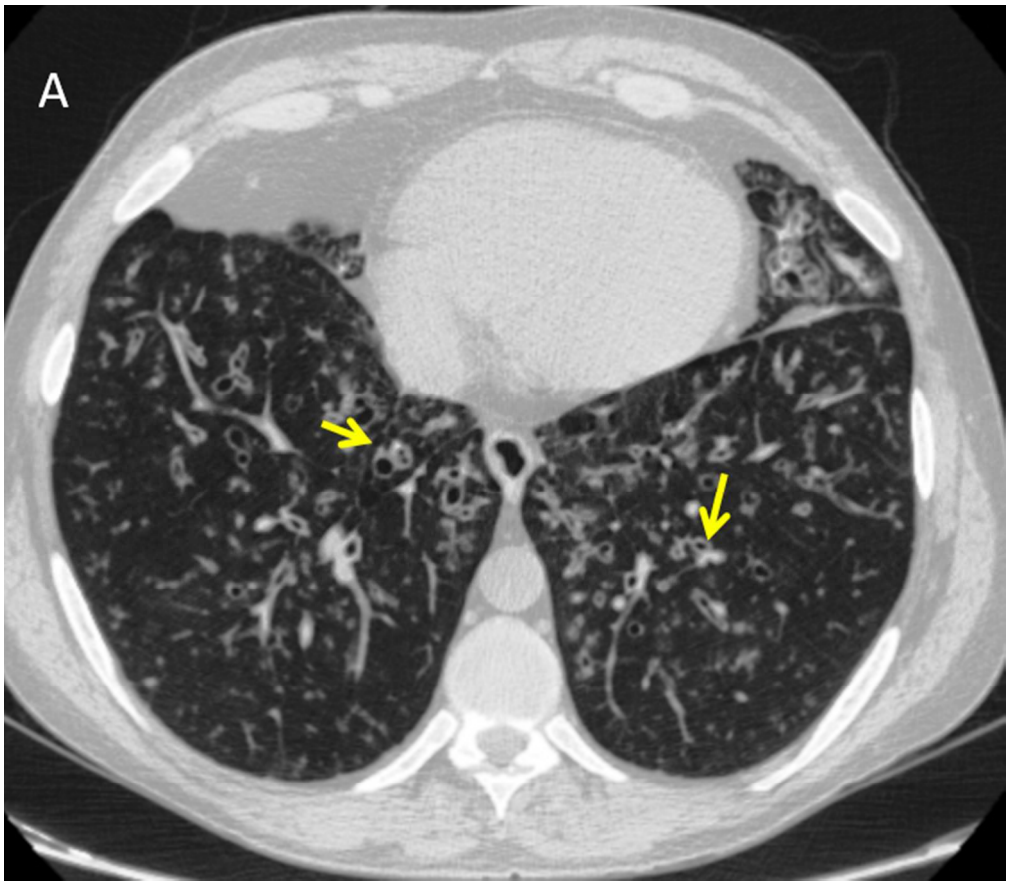
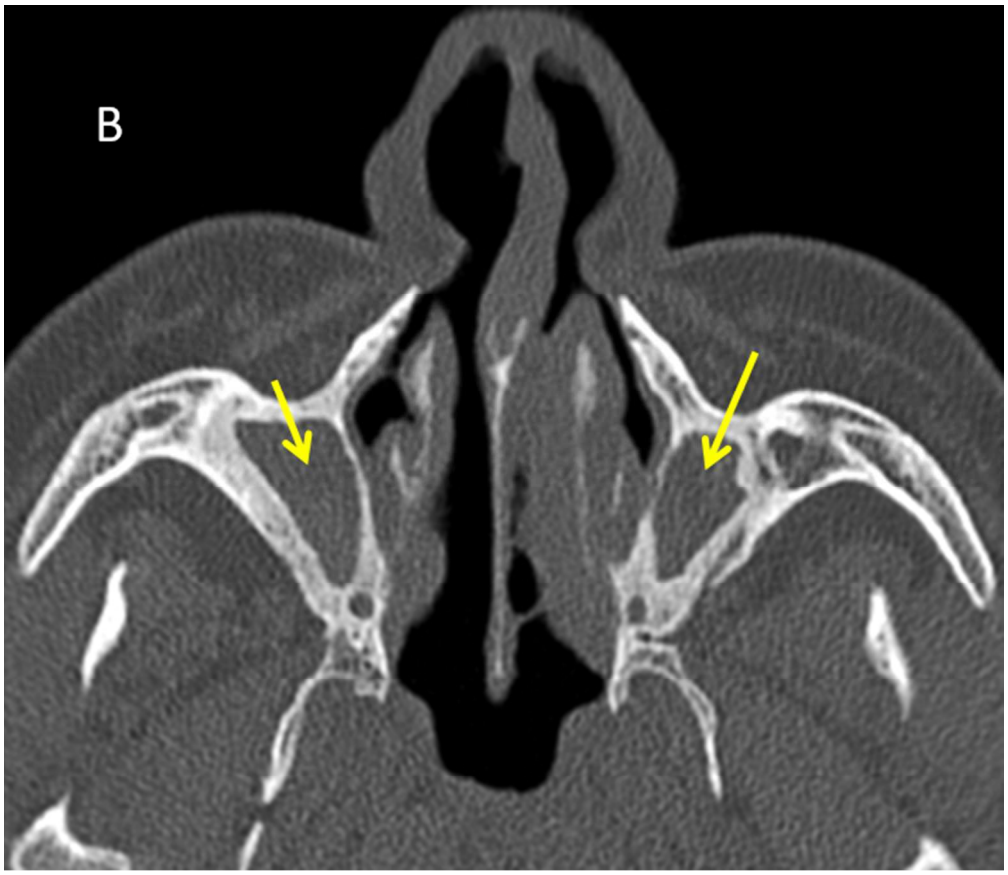
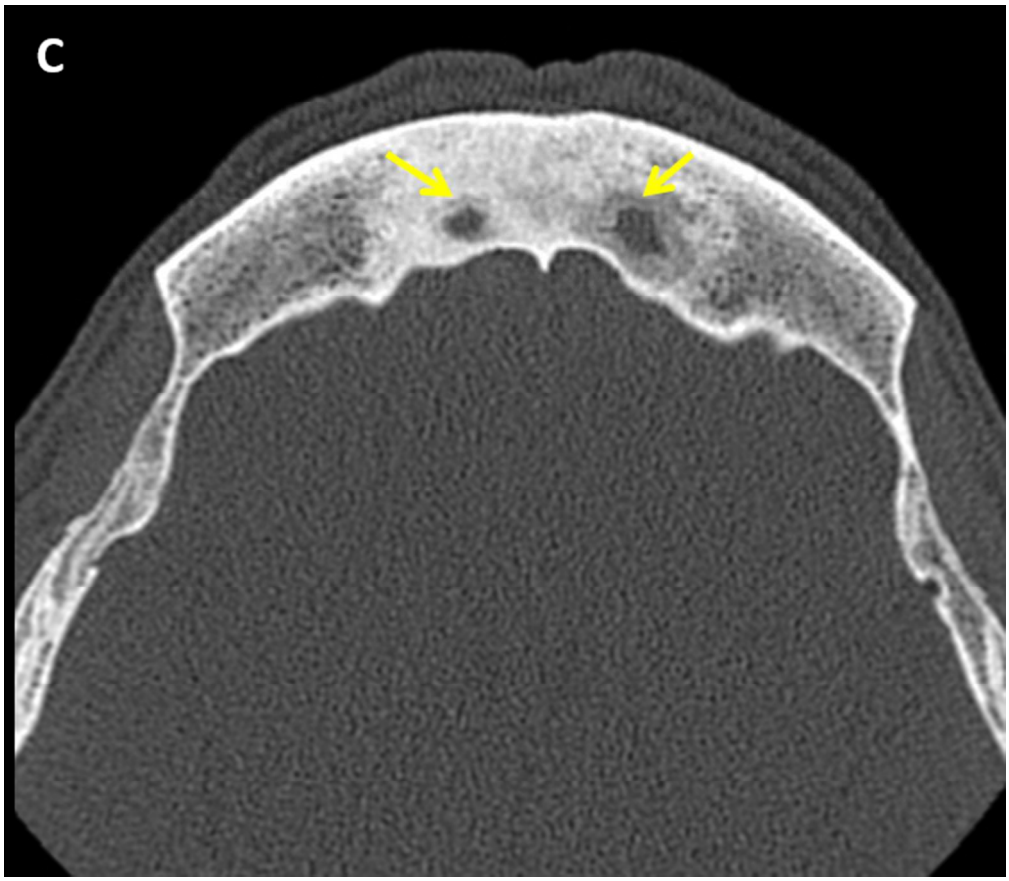


Rx typical findings in a patient with PCD: Bilateral bronchiectasis (arrows)



Rx typical findings in a patient with PCD: Hypoplasia of the maxillary sinuses and sinusitis (arrows)



Rx typical findings in a patient with PCD: Aplasia of the frontal sinuses (arrows)

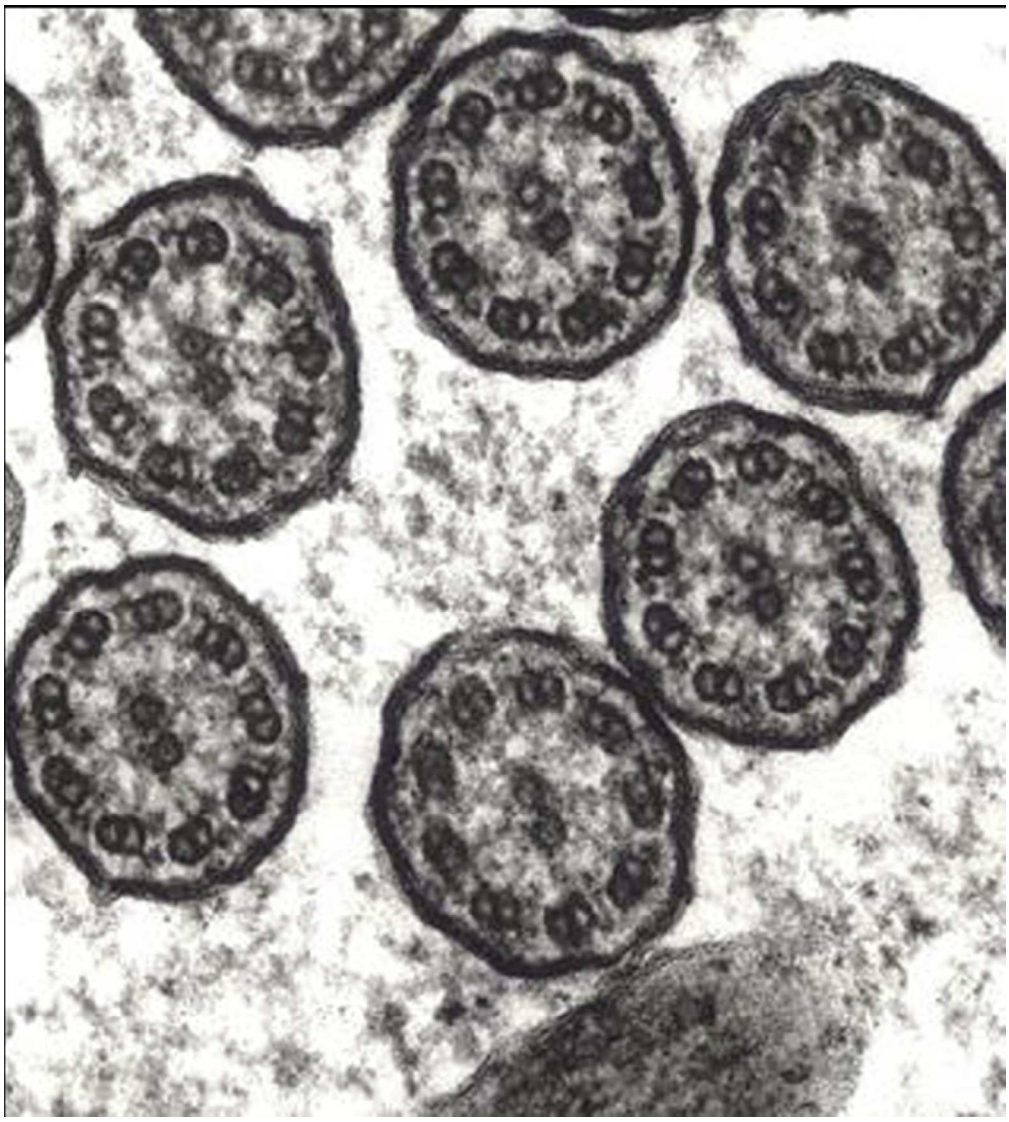


Figure 2A: Representative transmission electron microcopy picture of normal cilia ultrastructure.

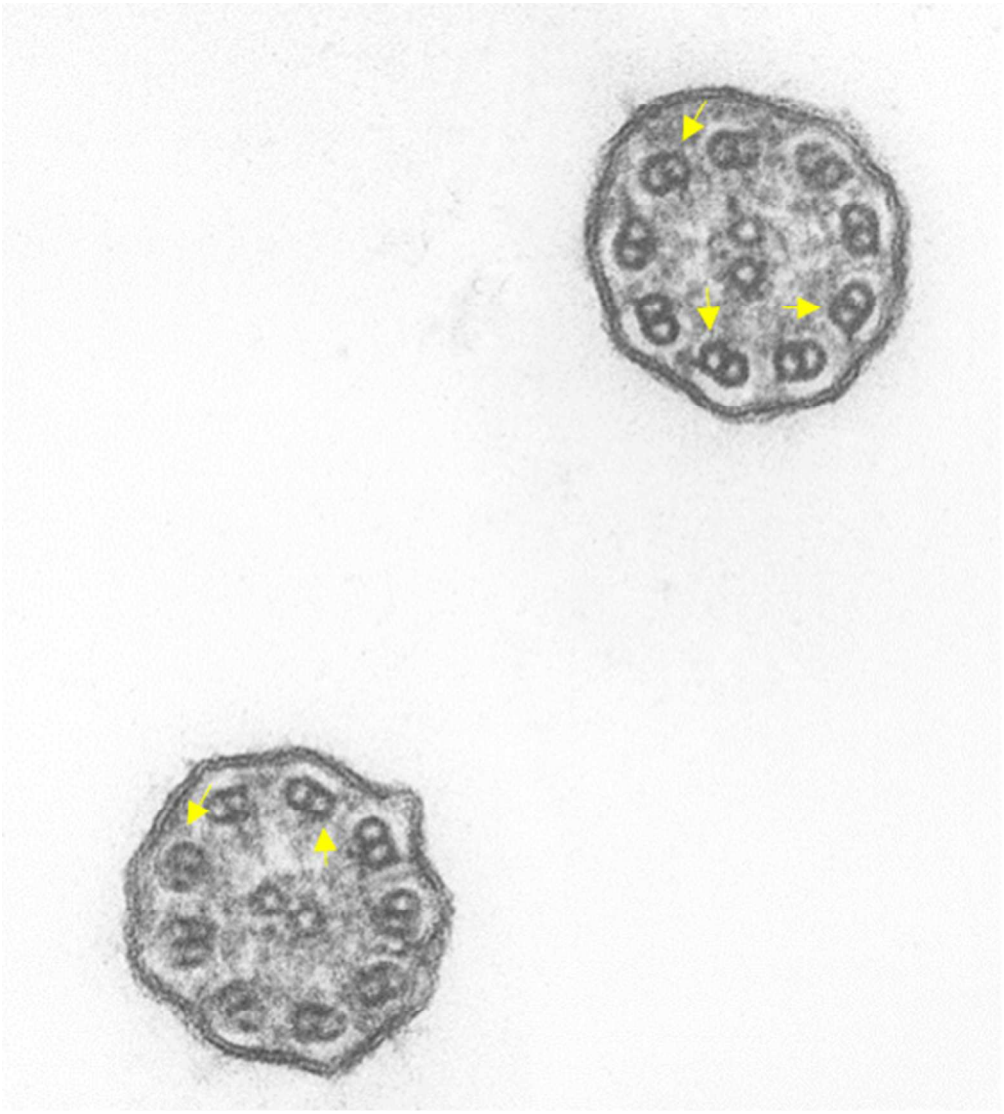


Figure 2A: Representative transmission electron microscopy picture of Primary Ciliary Dyskinesia dynein arms defects.

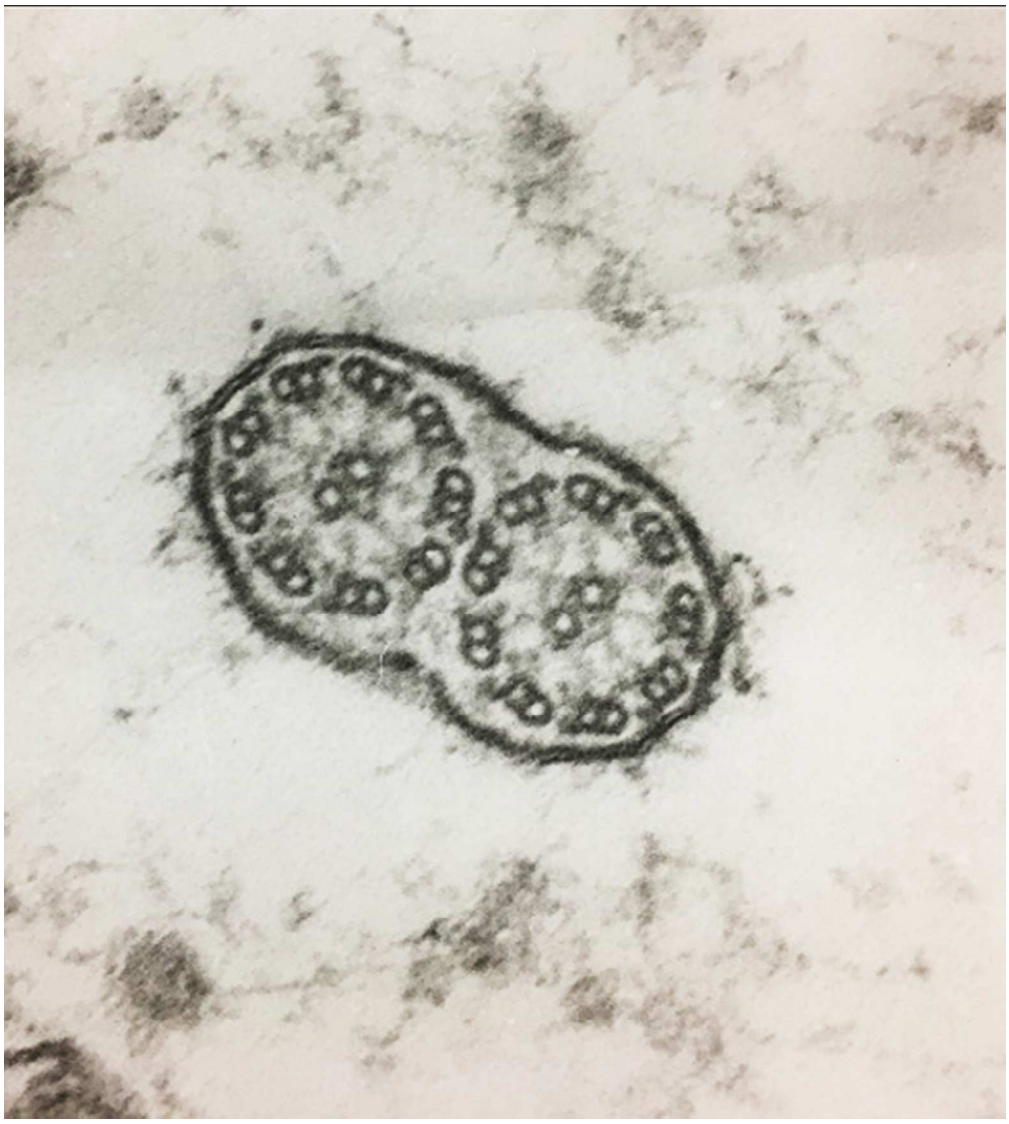


Figure 2C: Representative transmission electron microscopy picture of a cilia complex due to secondary defects, with normal ultrastructure in each cilia.

Organ	**Clinical manifestation**
Lung	Neonatal respiratory distress
	Recurrent infections
	Bronchiectasis
Ear	Secretory otitis media
	Chronic otitis media
	Hearing loss
Fossae and sinuses	Chronic sinusitis
	Hypoplasia of sinuses, especially the frontal
Genital-urinary tract	Male infertility
	Female: reduced fertility
Organic laterality	*Situs inversus totalis*
	Situs ambiguus (heterotaxia)
Central nervous system	Hydrocephalus (extremely rare)

Dear reviewers, we appreciate your feedback and believe that the suggested changes have been helpful in order to improve the review.

Editorial Comments

1. Please confirm that your figures / tables are original and have not previously been published, OR, provide a copy of permission to use.

All authors confirm that figures and tables are original and have not previously been published.

2. Please provide a few reference annotations (*=of importance, **= of considerable importance), followed by a brief sentence explaining why the reference is considered to be of interest.

Reference annotations including why they are considered to be of interest are included in the text. Changes are highlighted in yellow.

Referee(s)' Comments to Author:

Referee: 1

Comments to the Author
The authors have made a number of changes in this revised manuscript. As a result, the clarity and depth of the manuscript have been improved. There are a few minor issues remaining:

1. PAGES 8-9: The difference between screening tests and diagnostic tests for PCD needs further clarification. The authors added a statement that nasal NO cannot be used as a diagnostic test because of the association of false positives and false negatives, but false negative and false positive results are also a major problem for transmission electron microscopy, which is listed as a diagnostic tool. This remains a confusing point. It would be helpful if the authors stated their recommended scheme for PCD diagnosis, perhaps in the Expert Opinion section of the paper.

According to the last European Respiratory Society Task Force recommendations, we decided to include nNO levels into the diagnostic pathway (page 8). We also clarified that few PCD patients have normal levels, and low nNO levels are also associated with other similar pathologies as Cystic Fibrosis. Moreover, nNO levels cannot be measured in young children.

We also included a recommended scheme for PCD diagnosis in the Expert Opinion Section of the paper: “To diagnose PCD patients, having a strong clinical history (including nNO levels if it is possible), the starting point is HSVM analysis of the ciliary function, followed by TEM analysis of the ciliary structure. In case of poor health of the initial sample, ALI primary cultures of nasal or bronchial epithelial cells can be done, and ciliary function and structure re-analysed in order to differentiate primary from secondary defects. Sometimes it is necessary to repeat the sampling and tests, especially the motility studies. These tests are complex and it is essential that the studies are carried out by experts”.

Changes are highlighted in yellow.

2. PAGE 13, TABLE 2: The authors twice state on page 13 that there are 35 PCD genes, but Table 2 now has 38 genes plus an empty line with a reference for Ak7, which would presumably bring the total to 39. The numbers need to be updated, and the empty line in the table needs to be completed.

Reviewer is right, in the final version uploaded AK7 line was removed and we didn’t notice it. Now is completed and highlighted in yellow. The number of genes is also changed to 39 in the text, and changes are also highlighted in yellow.

3. FIGURE 2B: It would help the reader if the authors added arrows pointing to the dynein arm defects in the axonemes.

We agree with the reviewer. Now, arrows have been added to figure 2B pointing some dynein arm defects.

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