GENETIC DIAGNOSIS AND RESPIRATORY MANAGEMENT OF PRIMARY CILIARY DYSKINESIA

TAMARA PAFF

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Motile cilia line the entire respiratory tract and are responsible for "sweeping" the airways, thereby removing inhaled noxious substances and micro-organisms. By doing so these hair-like organelles are an important component of the body's innate immune system. Patients with an inherited defect in this ciliary motility consequently suffer from frequent upper and lower respiratory tract infections, eventually leading to permanent lung damage (i.e. bronchiectasis). Diagnosing primary ciliary dyskinesia (PCD) at an early stage is thought to improve pulmonary outcome later in life [1, 2]. As there is no single gold standard test, several tests that require expert skills to perform and to interpret need to be combined. In recent years, the wide-spread use of next-generation sequencing (NGS), allowing high-throughput sequencing of DNA, accelerated the discovery of PCD-related genes. These advances opened up the possibility of genetic testing in the diagnostic approach, requiring the establishment of its exact role.

The respiratory management of PCD patients is hampered by the lack of scientific evidence. As patients with cystic fibrosis (CF) suffer from similar pulmonary infections, management of PCD is primarily based on extrapolations from CF guidelines and personal experiences. This approach is understandable but questionable, as CF and PCD have distinct underlying pathophysiology. The aims of this thesis are (1) to gain more insight into the genetic background of Dutch PCD patients and the role of genetic testing in the diagnostic approach and (2) to gain more insight in the respiratory management of PCD. In **chapter 1** I aim to provide a complete overview of PCD characteristics, diagnostic methods and options for respiratory management to put the other chapters of this thesis into perspective.

HISTORY OF PRIMARY CILIARY DYSKINESIA

In 1904, Siewart (or Zivat, Zivert, Sivert) described a 21 year old with bronchiectasis associated with situs inversus (i.e. a complete mirror image of the internal organs) [3]. Kartagener later reported the triad of bronchiectasis, situs inversus and sinusitis in 1933, but it was not until the mid-1970s that Afzelius and Pedersen recognized infertility as a feature and proposed the unifying role of cilia to explain the syndrome [4–6]. Having noted absent dynein arms in the cilia of patients with the syndrome, Afzelius later demonstrated that the cilia were immotile, prompting the change of name from 'Kartagener's Syndrome' to 'Immotile Cilia Syndrome' [5]. These reports provided the evidence for assessment of the ciliary ultrastructure and motility as the basis of diagnostic testing. Recognition that outer dynein arm anomalies were not the only ultrastructural defect associated with the syndrome gave early insights into the underlying heterogeneity of the disorder [7, 8]. Following recognition that a number of patients had motile but dyskinetic cilia the name was further changed in the mid-1980s to 'primary ciliary dyskinesia' [9–12].

CLINICAL CHARACTERISTICS

PCD is a rare disease occurring in an estimated 1:10.000 – 1:30.000 newborns. It is important for clinicians to recognize phenotypic features of PCD to enable diagnosis at an early age [13, 14]. This can be a major challenge as some disease characteristics show overlap with more frequently occurring respiratory diseases in childhood, such as recurrent airway infections without an underlying disease, asthma, immune deficiencies, congenital malformations of the lungs and airways (like bronchomalacia) or even (mild) CF. In table 1 clinical features are summarized that should raise the suspicion of PCD and prompt referral to a specialized diagnostic center [15]. Symptoms can be classified by the organ systems in which cilia are present; the respiratory system, the embryonic node and the reproductive system.

Table 1. Who to refer for diagnostic testing.

Patients with early onset of recurrent respiratory tract symptoms and any of the following:	
1.	Situs inversus (SI) totalis or any heterotaxic syndrome (approximately 50% have normal situs)
2.	Neonatal nasal congestion and/ or unexplained neonatal distress in term infants
3.	Positive family history for PCD
4.	Males with dysmotile sperm
5.	Persistent productive cough/ bronchiectasis / severe upper airway after more common causes like allergies, asthma, immune deficiencies and CF have been excluded.
6.	Early onset of the combination of both severe upper and lower respiratory tract infections
7.	Persistent/ frequent intermittent serous otitis media (glue ear) associated with respiratory symptoms

Respiratory symptoms

Respiratory symptoms occur due to ineffective clearance of mucus. As cilia are present throughout the entire upper and lower airways, PCD patients often experience chronic nasal discharge, sinusitis, serous otitis media and pneumonia [16, 17]. Nasal congestion is usually present at the day of birth or shortly after and remains throughout life. Seventy-five to 85% of term neonates with PCD show neonatal respiratory distress due to lobar collapse or pneumonia, caused by ineffective clearance of fetal lung fluid [18, 19]. More than half of PCD patients have chronic sinusitis [20]. Serous otitis media, unlike in healthy children, often persists into adulthood [21, 22]. Early onset of wet sounding cough and recurrent lower airway infections occur in all PCD patients, leading to a 0.5%-1.5% decrease in forced expiratory volume in 1 second (FEV1) per year [23]. Frequently identified microorganisms in both children and adults are similar to CF; *Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae and Pseudomonas aeruginosa*. Although pulmonary disease progression is milder than in CF, half of the pediatric PCD patients and almost all adult patients develop bronchiectasis [24]. This occurs predominantly in the lingula, middle

and lower lobes. A significant percentage of patients (4-25%) can eventually experience respiratory failure, indicating that disease progression in some PCD patients is not as mild as often thought [1, 15].

Situs abnormalities and cardiac defects

Leftward flow created by the beating action of monocilia on the embryonic node triggers the left-right determination events of the internal organs [25, 26]. Theoretically there is a 50% chance of a complete mirror image of the internal organs when cilia are not functioning properly. A comprehensive overview of 305 PCD patients in the US showed that 41% of patients have situs inversus totalis and 12.1% have situs ambiguous (i.e. any other laterality defect other than situs inversus totalis), combined with a simple or complex cardiac defect in 2.3% and 2.6% of cases, respectively [27]. Polysplenia or asplenia occurs in more than half of patients with situs ambiguous, requiring protection against encapsulated bacteria [24, 27].

Fertility problems

As motile cilia share a common axonemal structure with spermatozoa flagella, PCD often leads to male sub- or infertility [4]. Female fertility varies from achieving pregnancy without any difficulties to ectopic pregnancies or subfertility, due to dysfunction of motile cilia in the fallopian tubes [1, 28–31]. Successful use of *in vitro* fertilization (IVF) treatment for male and female PCD patients has been reported [30, 32, 33].

Rare clinical manifestations of PCD

Other clinical manifestations of PCD are rare and less well understood. Hydrocephalus, likely reflecting dysfunctional ependymal cilia, is a common phenotype in mouse mutants with immotile cilia but is rarely seen in individuals with PCD [34–36]. Sporadically, PCD co-segregates with intellectual disability, retinitis pigmentosa or autosomal dominant polycystic kidney disease [37–39].

CURRENT DIAGNOSTIC APPROACHES

There is no single reference standard diagnostic test for PCD and diagnosis usually requires a number of technically demanding, sophisticated investigations [40]. As a result, clinical unawareness, under-diagnosis and diagnostic delay are problems in many countries [14]. One third of European PCD patients visited their doctor on more than 40 occasions before the diagnosis of PCD was considered [41]. The average age at diagnosis across Europe is 5.8 years in those without situs inversus and 3.5 years in those with, suggesting under-recognition in those with normal situs. The availability and combination of diagnostic tests vary between

countries [14]. Recently, the European Respiratory Society (ERS) guideline for the diagnosis of PCD presented a consensus as to which diagnostic results constitute a 'definite positive diagnosis', a 'highly likely diagnosis', an 'inconclusive diagnosis' or 'highly unlikely diagnosis' [42]. However, for many of the tests recommended there is no global agreement regarding the standardisation of conduct or reporting. According to these guidelines, a definite diagnosis is made by a hallmark ciliary ultrastructure defect evaluated by transmission electron microscopy (TEM) or non-ambiguous bi-allelic mutations in PCD-related genes. A highly likely diagnosis is made by a combination of low nasal nitric oxide (nNO) and a ciliary motility defect, evaluated by high-speed videomicroscopy analysis (HVMA). Independent of the exact diagnostic pathway chosen, performing these tests in an expert center has shown to positively influence the rate and timing of diagnosis in European countries [14, 42]. In immunofluorescence, ciliary proteins are labeled to determine their localization in respiratory epithelial cells. This technique is mainly used to improve understanding of the downstream effects of mutations in novel PCD-related genes. As it enables identification of ultrastructural abnormalities that are detectable by TEM and also in some cases where TEM is apparently normal or shows subtle defects, the technique may also be useful in the diagnostic work-up [43].

Medical history

Thorough evaluation of clinical history is a valuable tool in diagnosing PCD. PCD patients reported that in their opinion the most important reasons for their diagnostic delay was that clinicians did not take their symptoms seriously and took insufficient notice of their past medical history [41]. Characteristics that seem most predictive of having PCD are a laterality defect (odds ratio 7.7); unexplained neonatal respiratory distress (odds ratio 6.6); early onset, year-round nasal congestion (odds ratio 3.4) and early-onset, year-round wet cough (odds ratio 3.1) [19]. Currently, efforts are made by the international community to develop practical clinical tools to guide physicians in who they need to refer to an expertise center. As an example, the PICADAR tool consists of 7 simple questions leading to a score with individual probability of having a PCD diagnosis [44]. Upon external validation in a tertiary center the AUC reached 0.87. As the aim is to eventually use such tools to identify patients for referral from primary or secondary care to a tertiary diagnostic center, this will require further validation in this setting.

Nasal nitric oxide

Nitric oxide, which is produced throughout the airways but most abundantly in the nasal sinuses, is markedly reduced in most PCD patients. Although the underlying mechanism of this phenomenon is still unknown, nNO measurements can separate PCD patients from healthy controls and other respiratory diseases [45]. That said, some overlap in low

nNO concentration between patients with chronic rhinosinusitis, CF and PCD is observed, suggesting that disorders obstructing the nasal passage may falsely lower nNO concentration [46–48]. Large-scale screening of referred patients for PCD diagnostics in the US and Europe has shown that when standardized protocols are used (velum-closure, chemiluminescence analyzer), a sensitivity of 90-100% and specificity of 75-97% can be reached with cut-off values between 30-82 nL/min [42, 45, 48–50]. This may suggest that nNO measurements can be helpful as a screening tool, especially in secondary care centers, where other diagnostic techniques are unavailable. However, such validation studies have not been performed yet. When interpreting nNO results it is important to realize its limitations. Measurement in young children is possible, but discrimination is reduced, as nNO is inversely proportional to age in healthy subjects <12 years. Further, specificity can be unacceptably low in small children that are unable to perform a breath-holding procedure. There was a false positive rate of 39% in children <6 years in which an alternative tidal breathing maneuver was used [48]. If nNO screening becomes more widely used in secondary centers, we should be aware of the effect this has on the positive predictive value (figure 1) [51]. Up to 9% of PCD patients have normal nNO [48, 52, 53]. There is increasing evidence that some genetic mutations are associated with a more subtle beating abnormality and nNO levels in the normal range [54–56]. In summary, nNO should only be used for screening in suspect cases to avoid overwhelming of diagnostic services and should not be used to rule out PCD if clinical suspicion is high.

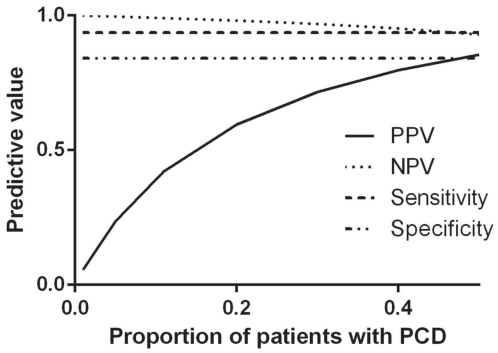

Figure 1. Relationship between positive predictive value (PPV), negative predictive value (NPV) and the pretest probability of PCD (proportion of patients with PCD in the tested population) [51].

High-speed videomicroscopy

In order to evaluate ciliary motility and ultrastructure, a good quality epithelial sample is obtained from the upper or lower airways by a trained health care professional. Nasal samples are most easily obtained, but if the patient is having a bronchoscopy for other reasons, lower airway samples can be taken [57]. Ciliary motion is evaluated by high-

speed videomicroscopy, which allows determination of both ciliary beat frequency (CBF) and ciliary beat pattern (CBP). Abnormalities of CBF and CBP can also occur secondary to infection, damage during sampling or inflammation of the epithelia cells complicating the diagnostic picture. Following abnormal analysis it is therefore necessary to reanalyse CBF and CBP following culture of the epithelial cells or following a repeat brushing to confirm that abnormalities are due to a congenital defect. Whilst direct measurement of CBP and CBF using HVMA is generally considered the most accurate and reproducible technique, it is time consuming and incurs risk of operator error due to selection bias. Several groups have attempted to overcome these problems by developing software to automate analysis from the digital images [58–60].

Transmission electron microscopy

TEM allows visualization of the ultrastructure of ciliary axonemes, which contain more than 200 proteins (figure 2A and B). Normal cilia have a structure of nine peripheral microtubular doublets and a central pair (9+2 arrangement). The accessory axonemal components are the outer dynein arms (ODA), inner dynein arms (IDA), radial spokes and the nexin-dynein regulatory complex (N-DRC). Dynein arms contain adenosine triphosphatases and act as motors to achieve ciliary motion by sliding of adjacent microtubular doublets. The most common ultrastructural defects in PCD are: ODA-defects (~25-50%) and combined IDA- and ODA-defects (~25-50%) [47, 57-59]. IDA defects associated with microtubular disorganisation occur in ~15% of PCD, but isolated IDA defects as a cause of PCD are controversial particularly as no mutations have been identified in IDA proteins. IDA are difficult to identify due to the decreased repeats along the ciliary axoneme compared to the ODA (figure 2C) therefore false positive IDA defects are likely [61]. Central pair defects occur less frequently (~5-15%) and are associated with a mix of both normal and abnormal cilia, therefore adequate numbers of cilia need to be viewed [62, 63]. Previously TEM was considered the "gold standard" but it is now recognized that 20-30% of PCD patients have normal ultrastructure when analyzed by TEM [62, 64]. An additional limitation of TEM is that inflammation and infection, can alter the normal 9+2 arrangement [65]. Similar problems can occur if cells are poorly fixed. Novel research tools that provide 3D visualization of the ciliary ultrastructure have revealed more subtle defects. As an example, electron tomography demonstrated ultrastructural defects in PCD patients with *DNAH11* and *HYDIN* mutations, who did not appear to have defects on classic TEM [66].

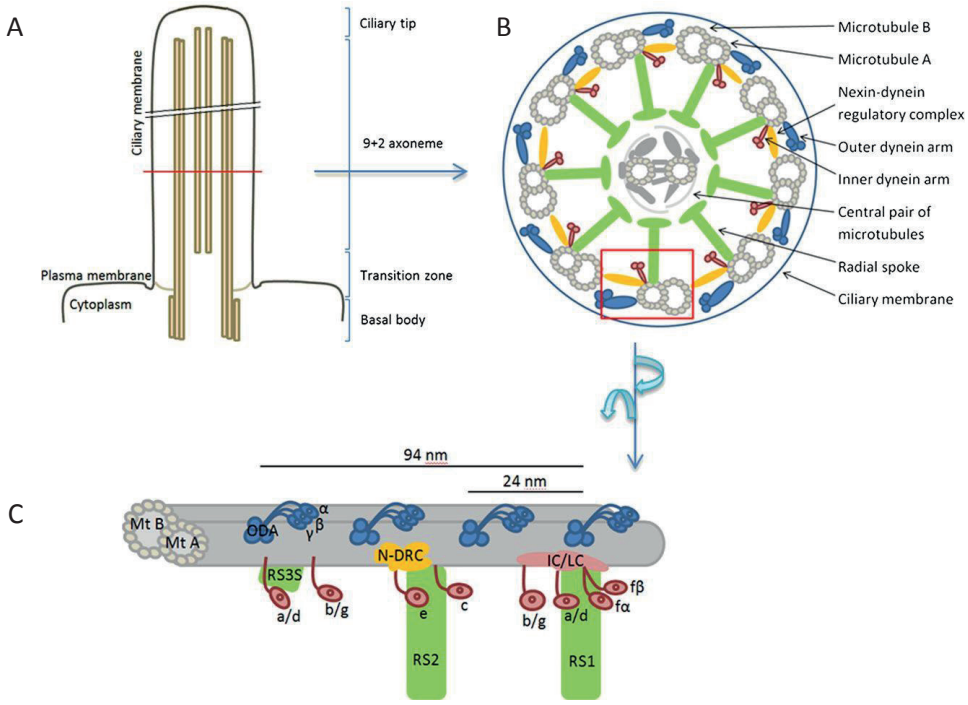

Figure 2. Schematic diagram of the ciliary axoneme.

A, Longitudinal section of a cilium. B, The cross-section (at the site of the red line in 2A) shows 9 peripheral microtubule doublets surrounding a central pair. C, ODAs are present every 24 nm and all others structures are present less frequently, every 96 nm [67].

Genetics

Obtaining multiple epithelial samples for HVMA and TEM analysis can be invasive, especially in children. Moreover, these tests are technically challenging, labor-intensive and require expert skills that are not readily available in every country. In contrast, DNA can be obtained from saliva or blood that can easily be transported to any laboratory. Genetic testing in PCD may therefore aid in preventing diagnostic delay. An important hurdle to overcome in this process is identifying all genes that are related to PCD. Currently, roughly two-third of all PCD-associated genes are known [68].

PCD is usually inherited in an autosomal recessive manner, but in rare instances other modes of inheritance such as X-linked or autosomal dominance have been reported, exclusively with syndromic co-segregation [37–39, 69]. In recent years, rapid advances have been made in the understanding of the heterogeneous molecular basis of PCD. Linkage analysis and candidate gene approaches were used to identify the first PCD-related genes *DNAH5* and *DNAI1* [70, 71]. These early studies focused on identification of genes encoding recognizable ultrastructural components such as the ODA. It was not until the widespread use of NGS that

the majority of PCD genes was identified [68]. This technique allows DNA sequencing of hundreds of short reads simultaneously and maps them to the reference genome to identify a person's variants. By sequencing a panel of preselected candidate genes (i.e. targeted-exome sequencing) or the entire protein-coding region of our genome (i.e. whole-exome sequencing, or WES) in families with PCD, the total number of currently known PCD-related genes has increased to 36. This has led to several important discoveries. For the first time, genes were linked to PCD that encode proteins involved in the (pre-) assembly, transport and docking of ciliary axonemal components. Second, novel gene defects were identified in patients with a typical PCD phenotype but with (near) normal ciliary ultrastructure or CBP [72, 73]. Genetic characterization has thus become increasingly important to confirm diagnosis in these patients who would otherwise have inconclusive results or a false negative diagnosis [42].

Now the discovery of PCD-associated genes has enabled genetic characterization of many patients, it is to be determined what the exact role of genetic testing in the diagnostic approach should be. One-by-one screening of genes in such a genetically heterogeneous disorder is not cost-effective and very time consuming. Alternatively, whole-exome sequencing enables identification of mutations in PCD-related genes as well as in undiscovered PCD-related genes. This technique is still primarily reserved for the research field as there are some important challenges to be faced before implementation into the diagnostic algorithm [74]. These include processing of the huge volume of data that is generated for every individual, how to deal with incomplete coverage of many parts of the exome that are difficult to sequence (low reading depth) and the analysis of variants with unknown significance. Using a targeted-exome panel has the advantage of reaching better coverage, having less data to analyze and no incidental findings. A complete overview of the genes that are currently linked to PCD is given in table 2 and includes the corresponding ultrastructural and motility defect.

Genotype-phenotype

The mechanisms responsible for determining clinical phenotypes in mendelian diseases are still not well understood. Specific disease genotypes, epigentic and environmental influences are expected to play an important role. In PCD, the association between genetic defects and the clinical phenotype is largely unknown. International collaborations are developing large meta-cohorts to ensure sufficient numbers of patients with mutations in each PCD-associated gene. Several genotype-phenotype relations have been described, but caution needs to be kept as some descriptions are based on low numbers of patients. Mutations causing reduced generation of multiple motile cilia (RGMC; MCIDAS, CCNO genes) and those causing IDA defects with microtubular disorganization (CCDC39, CCDC40) have been reported to result in relatively severe lung disease [104, 105, 108, 109].

Table 2. Overview of genes currently linked to primary ciliary dyskinesia.

Gene	Protein class	Ciliary ultrastructural defect by TEM	Ciliary motion defect by HSVM	Clinical phenotype	Reference
DNAH5	ODA-HC	Absent ODAs	Immotile cilia with occasional stiff cilia	Classic	[70]
DNAH11	ODA-HC	No defect	Stiff hyperkinetic cilia and cilia with low CBF/immotility	Classic	[72, 75]
DNAI1	ODA-IC	Absent ODAs	Stiff and immotile cilia	Classic	[71, 76]
DNAI2	ODA-IC	Absent ODAs	Immotile cilia	Classic	[77]
NME8	ODA-IC/LC	Absent or shortened ODAs	Normal to immotile cilia	Classic	[78]
DNAL1	ODA-LC	Absent or shortened ODAs	Low CBF	Classic	[79]
CCDC39	IDA and N-DRC	Absent IDAs and MTD	Fast, flickery and stiff cilia	Classic.	[80]
CCDC40	IDA and N-DRC	Absent IDAs and MTD	Fast, flickery and stiff cilia	Classic.	[81]
CCDC65	N-DRC	Absent IDAs and MTD	Stiff and dyskinetic cilia	No situs inversus	[82]
DRC1 (CCDC164)	N-DRC	Absent N-DRC. Single tubuli	Hyperkinetic and stiff cilia	No situs inversus	[83]
GAS8	N-DRC	Subtle misalignment of outer doublets	Subtle reduction of ciliary amplitude	Classic	[56]
RSPH1	RS	Ciliary transposition	Low CBF/immotile cilia and stiff cilia with normal CBF	No situs inversus	[54, 55]
RSPH3	RS	Near absent RS and variable CC defects	Stiff and immotile cilia	No situs inversus	[84]
RSPH4A	RS	Ciliary transposition	Low CBF/immotile cilia and cilia with circular pattern and normal CBF	No situs inversus	[85]
RSPH9	RS	Ciliary transposition	Low CBF/immotile cilia and cilia with circular pattern and normal CBF	No situs inversus	[85]

Table 2. Overview of genes currently linked to primary ciliary dyskinesia. *(Continued)*

Gene	Protein class	Ciliary ultrastructural defect by TEM	Ciliary motion defect by HSVM	Clinical phenotype	Reference
DNAJB13	RS/CP	Absent CP	Stiff cilia	No situs inversus	[86]
HYDIN	CP	CP defect, occasionally ciliary transposition	Stiff cilia with lack of coordination. Occasionally immotile cilia	No situs inversus	[73]
TTC25	ODA docking protein	Absent ODAs	Immotile cilia or cilia with some residual flickery movement	Classic	[87]
CCDC103	DA anchoring protein	Absent ODAs + IDAs	Stiff cilia with lack of coordination or immotile cilia	Classic	[88]
CCDC114	ODA docking protein	Absent ODAs + IDAs	Immotile cilia. Occasionally some twitching cilia	Normal male fertility	[89, 90]
ARMC4	(Pre-) assembly/ transport protein	Absent ODAs	Low CBF and stiff or immotile cilia	Classic	[91]
C21orf59	(Pre-) assembly protein	Absent ODAs + IDAs	Immotile cilia	Classic	[82]
CCDC151	(Pre-)assembly protein	Absent ODAs	Immotile cilia	Classic	[92]
LRRC6	(Pre-) assembly protein	Absent ODAs + IDAs	Immotile cilia	Classic	[93]
PIH1D3	(Pre-)assembly protein	Absent ODAs + reduced to absent IDAs	Immotile cilia	Classic	[94]
SPAG1	(Pre-) assembly protein	Absent ODAs + IDAs	Stiff and immotile cilia	Classic	[95]
ZMYND10	(Pre-) assembly protein	Absent ODAs + IDAs	Low CBF and stiff or immotile cilia	Classic	[96, 97]
DNAAF1 (LRRC50)	(Pre-) assembly protein	Absent ODAs + IDAs	Immotile cilia	Classic	[98, 99]
DNAAF2 (KTU)	(Pre-) assembly protein	Absent ODAs + IDAs	Immotile cilia	Classic	[100]
DNAAF3	(Pre-) assembly protein	Absent ODAs + IDAs	Immotile cilia	Classic	[101]

Table 2. Overview of genes currently linked to primary ciliary dyskinesia. *(Continued)*

Gene	Protein class	Ciliary ultrastructural defect by TEM	Ciliary motion defect by HSVM	Clinical phenotype	Reference
DNAAF4 (DYX1C1)	(Pre-) assembly protein	Absent ODAs + IDAs	Low CBF and immotile cilia	Classic	[102]
DNAAF5 (HEATR2)	(Pre-) assembly protein	Absent ODAs + IDAs	Stiff and immotile cilia	Classic	[103]
CCNO	Multiciliated cell differentiation protein	RGMC	No defect	No situs inversus	[104]
MCIDAS	Multiciliated cell differentiation protein	RGMC	Immotile cilia	No situs inversus	[105]
RPGR	NA	Disturbed ciliary orientation	Uncoordinated cilia	Retinitis pigmentosa	[106, 107]
OFD1	NA	NA	Dyskinetic cilia (no details described)	Macrocephaly, mental retardation	[39]

Abbreviaties: ODA, outer dynein arm; HC, heavy chain; IC, intermediate chain; LC, light chain; IDA, inner dynein arm; N-DRC, nexin-dynein regulatory complex; RS, radial spokes; CP, central pair; DA, dynein arm; TEM, transmission electron microscopy; MTD, microtubule disorganization; RGMC, reduced generation of multiple motile cilia; HSVM, high-speed videomicroscopy; CBF, ciliary beat frequency.

In contrast, mutations in RSPH1 (a mutation in a gene coding for one of the radial spoke subunits) reportedly cause a mild phenotype [110]. Situs abnormalities are not observed in patients with mutations affecting the central pair or radial spokes [54, 55, 85], nor in patients with reduced generation of multiple motile cilia [104, 105]. Varying severity of the clinical course between family members and between patients with similar gene defects has raised the question if modifier genes play a role in the clinical heterogeneity. However, due to the rare nature of the disease and the many genes that are related to PCD, unraveling the factors influencing clinical heterogeneity in PCD will be extremely challenging.

RESPIRATORY MANAGEMENT

A defective mucociliary system increases an individual's susceptibility to microbial colonization. Microbial colonization of the airways subsequently triggers a chronic inflammatory response that eventually leads to permanent bronchial wall thickening and dilatation (i.e. bronchiectasis). These changes in airway structure further contribute to mucus stasis and the self-perpetuating cycle of infection and inflammation [111]. This process generally evolves much slower in PCD than in CF, in which mucociliary clearance is altered due to a dehydrated mucous layer. In PCD a lung function decline of 0,5-1,5% FEV1 per year is observed, in contrast to 1,5-3,0% in CF [109, 112]. Although life expectancy is generally believed to be normal in PCD, respiratory failure does occur in a significant proportion of patients (4-25%), requiring lung transplantation [1, 15]. Due to the rarity of the disease there is a lack of evidence on how to manage the frequently occurring airway infections in PCD. Therefore, treatment practices are primarily based on personal experiences and extrapolations from CF care [15]. This approach is understandable as CF patients have similar airway infections and implemented treatment strategies have majorly contributed to the increased life expectancy in the last decades [113]. However, both diseases have distinct underlying pathology and the use of beneficial treatments in CF can be harmful in non-bronchiectasis patients [114]. Monitoring disease progression is hampered by the lack of sensitive clinical outcome parameters and unified patient registry. Recently, the first online international patient registry has been launched by the FP-7 BEST-CILIA network [43]. This will aid in investigating factors influencing disease progression and can be a platform for designing large RCTs to evaluate therapeutics. The current cornerstones of respiratory management of PCD are improving airway clearance and intensive monitoring of infections and aggressive antibiotic treatment.

Airway clearance

Patients are recommended to mobilize sputum by daily exercise, active cycle breathing and/or physiotherapy. Airway clearance is depended on a combination of coughing, ciliary

beating, the volume of the airway surface layer (ASL) and rheological properties of mucus (figure 3).

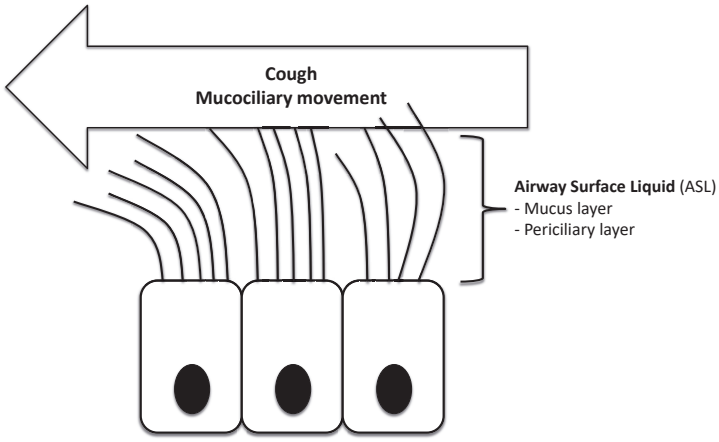

Figure 3. Schematic overview of airway epithelium and mucociliary clearance.

In PCD there is an underlying inherited defect in ciliary motility that cannot be restored. Therefore, therapeutic strategies are primarily aimed at increasing the ASL, lowering sputum viscosity and improving cough clearance. There are several techniques available that are frequently used in CF care. None, however, have been properly researched in PCD. First, mucous viscosity may be improved by nebulizations with a mucolytic agent, such as recombinant human DNAse I (rhDNAse) or N-acetylcysteine (NAC). RhDNAse cleaves extracellular DNA and provides improvement in lung function and a reduction in exacerbations in CF, by decreasing mucous viscosity [115]. Thus far it is not recommended in PCD, as an unexplained increase in pulmonary exacerbations was observed in non-CF bronchiectasis patients [114]. NAC, a sulfhydryl compound that breaks disulfide bonds, decreases sputum viscosity but nebulizations are usually not tolerated because of the sulfur taste. Oral application of NAC during three months in a small group of 13 PCD patients showed no clinical improvement [116]. Second, hyperosmolar agents, such as uridine-5'-triphosphate (UTP), mannitol and hypertonic saline (HS) attract water across the epithelial cells to increase the ASL and improve viscosity. In an early pilot study in 12 PCD patients nebulized UTP improved whole lung clearance during voluntary cough [117]. Mannitol increases time to new exacerbation and decreases mucus plugging in non-CF bronchiectasis and can have slight improvement in lung function in CF [118, 119]. Despite the lack of evidence, hypertonic saline is the only mucoactive agent that is regularly prescribed to PCD patients by physicians in the international community [15]. In CF patients HS rehydrates the airway mucous and bi-daily nebulizations have shown to significantly increase quality of life

and prolong the time to a new exacerbation [120]. As PCD and CF sputum seems to have similar biophysical properties, HS may improve transportability during coughing and regular physical activity in PCD patients [121].

Early recognition and treatment of airway infections

A pulmonary exacerbation (i.e. an episode of acute worsening of respiratory symptoms) is a critical event in the disease course. In CF it has been demonstrated that half of lung function decline is attributed to severe pulmonary infections requiring intravenous antibiotics [122]. Frequent exacerbations and also a short interval between consecutive exacerbations (< 6 months) leads to steeper decline in lung function over time [122–124]. Lifelong microbial surveillance is therefore aimed at swift recognition and aggressive treatment of infections. This strategy is thought to delay the onset of chronic infections and subsequent structural changes in the airways. However, huge variability exists in the definition of a pulmonary exacerbation and the exact treatment approach among clinicians and researchers [125]. Generally, microbial surveillance relies upon sputum culture for species determination and antibiotic sensitivity. Exacerbations are treated with empiric antibiotics aiming to treat the most commonly occurring pathogens in PCD such as *H. influenzae, S. aureus, S. pneumoniae and P. aeruginosa*, or treatment is guided by previously detected pathogens in sputum [15]. This approach has several limitations. First, obtaining a sputum specimen can be challenging, specifically in children with PCD that often have minimal sputum production or lack the technique to expectorate. Alternatively, less accurate methods such as a throat or cough swab are used or more invasive techniques such as a broncho-alveolar lavage. Second, bacterial cultures may take days to become positive, introducing a treatment delay. Third, the application of culture-independent techniques (i.e. microbiome analysis by RNA sequencing) recently revealed that a large part of the microbes that are present in the airways are not detected by using culture-based systems [126, 127]. Fourth, there is emerging evidence that respiratory viruses are associated with deterioration of pulmonary function and facilitation of bacterial colonization [128]. Taking this into account, monitoring of lung disease in CF and PCD patients may call for a more unbiased, personalized and non-invasive approach to recognize the entire spectrum of microbial, viral and/or inflammatory changes at an early stage.

The potential of exhaled breath analysis in monitoring of lung disease

In ancient times physicians already recognized that certain breath odors were associated with a specific pathological status. For example, patients with diabetes had a fruity smell, owing to the acetone and patients with renal failure had a particular fishy smell owing to uremia. To identify a certain smell our nose uses pattern recognition of exhaled volatile organic compounds (VOCs). VOCs are gaseous organic molecules that are emitted from the

fluid phase because they are highly volatile. Human VOCs are derived from many metabolic pathways and are released from skin, with feces, urine, and breath. Since cellular metabolism is altered by disease, the resulting change in VOCs may serve as biomarkers for particular pathophysiological conditions. In pulmonary medicine, breath is of special interest because of its intensive contact with the respiratory tract. Exhaled VOCs can be of local, systemic or exogenic origin. Many techniques for analyzing exhaled breath are currently available. Chemical analytical techniques allow identification of specific compounds. At the other end of the spectrum are pattern recognition based techniques (i.e. electronic noses) allowing probabilistic discrimination of biomarker profiles, without the identification of specific VOCs. It is possible to discriminate breath from healthy subjects from the breath of patients with various pulmonary diseases, such as asthma, COPD and ventilator-acquired pneumonia (VAP). These distinct breath patterns reflect differences in the underlining disease processes that can be used for diagnostic purposes. Additionally, it may be possible to identify a temporary change in disease status such as an infective pulmonary exacerbation. As an example, an electronic nose was able to detect a VAP in critically ill ICU patients [129]. Most likely such a changing breath pattern is the results of a combination of a host response and microbial changes. In vitro headspace analysis of bacterial cell cultures allows recognition of bacteria-specific VOCs that are not produced by the human body itself [130]. Some of these VOCs can also be found in human breath at the time of an infection. For instance, hydrogen cyanide is detected in exhaled breath of CF patients with a *Pseudomonas aeruginosa* infection [131]. But, irrespective of the responsible pathogen, CF patients with an exacerbation show lower levels of isoprene and higher levels of pentane [132, 133]. This may suggest that exhaled breath analysis can be a useful unbiased tool in the follow-up of patients with recurrent pulmonary exacerbations. If a pulmonary exacerbation could be detected at an early stage, even before a patient starts showing clinical symptoms, this could be of use in the monitoring and treatment of CF and PCD.

OUTLINE OF THE THESIS

The aim of this thesis is to study the genetic background and the respiratory management of primary ciliary dyskinesia, together with my co-workers. In part I of this thesis we first aim to give a state of the art overview of PCD characteristics, diagnostic methods, genetics and management (**this chapter**).

In part I, which includes chapters 2-4, we aim to describe the genetic background of Dutch PCD patients in more detail. Genetic characterization of PCD patients is pivotal to move the field of genetic testing forward. In **chapter 2**, we aim to identify the gene defect in PCD patients originating from the town of Volendam in the Netherlands. This area has historically

been isolated due to geographical and religious reasons and therefore has a different genetic make-up than the rest of the Netherlands. As a result, there is a much higher incidence of PCD in newborns from Volendam than in newborns from the rest of the Netherlands. This study can help identify a possible founder mutation in this PCD population.

In **chapter 3**, we aim to study the genetic background of a novel recessive X-linked form of PCD in three patients of a Dutch family and one patient of a German family. These PCD cases are the first ones described with an X-linked mode of inheritance without syndromal cosegregation. A different form of inheritance has important implications for the analysis of sequencing data and the genetic counseling of patients. This study is thus important in raising international awareness of the possibility of a recessive X-linked mode of inheritance in PCD patients. Identifying the gene defect in the families included in this study could aid in identifying the gene defect in other unsolved male cases.

In **chapter 4**, we aim to describe the genetic defects of a Dutch PCD cohort of 74 patients using a targeted gene panel of 26 PCD-related genes and 284 candidate genes. We use *in vitro* ciliogenesis experiments to prioritize candidate genes that show significant upregulation during the development of cilia. By screening a Dutch PCD cohort with a targeted gene panel we evaluate the diagnostic yield of a genetic test. This study is important in determining the distribution of genetic defects as this may vary enormously among countries. This may aid in establishing the best way to implement a genetic test in the Netherlands in the future.

In part II, including chapters 5-7, we investigate several aspects of respiratory management of PCD patients. In **chapter 5,** we aim to give an overview of the rationale and application of exhaled breath analysis in lung diseases so far.

In **chapter 6**, we aim to assess the potential of exhaled breath analysis in differentiating two mucociliary clearance disorders; PCD and CF. This study is a first step in determining whether exhaled metabolites reflect the differences in the underlying disease processes. To further investigate whether this technique may have potential in monitoring disease activity, we also compared patients with and without a pulmonary exacerbation.

In **chapter 7,** we aim to explore the effect of inhaled hypertonic saline on quality of life in adult PCD patients. We performed a randomised controlled crossover trial to observe changes in St. George's Respiratory Questionnaire scores and Quality of Life Bronchiectasis questionnaire scores in adult PCD patients. Additionally, we explored the effect on spirometry, pulmonary exacerbations and sputum and systemic inflammatory markers. In **chapter 8** the results of this thesis are discussed and directions for future research are given.

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SPLICE SITE MUTATIONS IN THE AXONEMAL OUTER DYNEIN ARM DOCKING COMPLEX GENE *CCDC114* CAUSE PRIMARY CILIARY DYSKINESIA

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ABSTRACT

Defects in motile cilia and sperm flagella cause primary ciliary dyskinesia (PCD), characterized by chronic airway disease, infertility, and left-right laterality disturbances, usually as a result of loss of the outer dynein arms (ODAs) that power cilia/flagella beating. Here, we identify loss-of-function mutations in *CCDC114* causing PCD with laterality malformations involving complex heart defects. CCDC114 is homologous to DCC2, an ODA microtubule-docking complex component of the biflagellate alga *Chlamydomonas*. We show that CCDC114 localizes along the entire length of human cilia and that its deficiency causes a complete absence of ciliary ODAs, resulting in immotile cilia. Thus, CCDC114 is an essential ciliary protein required for microtubular attachment of ODAs in the axoneme. Fertility is apparently not greatly affected by CCDC114 deficiency, and qPCR shows that this may explained by low transcript expression in testis compared to ciliated respiratory epithelium. One *CCDC114* mutation, c.742G>A, dating back to at least the 1400s, presents an important diagnostic and therapeutic target in the isolated Dutch Volendam population.

Motile cilia are found on the epithelial surface of the upper and lower respiratory airway systems, the brain ependyma and fallopian tubes. Their core structure (axoneme), shared with sperm flagella, comprises nine peripheral outer doublet microtubules surrounding a central microtubular pair ('9+2' arrangement), except in the case of motile embryonic node cilia that lack the central pair ('9+0'). Microtubule-associated protein complexes are attached along its length at regularly repeating intervals, which contribute to axonemal stability and the coordinated beating movement of cilia/flagella. These include paired inner and outer dynein arms (IDA and ODA) dynein motor protein complexes that provide the ATP-driven force for self-propagating axonemal beating, in addition to radial spoke complexes and nexin-dynein regulatory complexes [1]. In the biflagellate alga *Chlamydomonas*, a well-established model organism for human ciliary motility research because of its highly similar axonemal structure, the outer dynein arms are preassembled in the cytoplasm, transported to the axoneme, and then attached to the axonemal microtubules via outer dynein arm docking complexes [2, 3].

Primary ciliary dyskinesia (PCD [MIM 244400]) is a recessively inherited ciliary disorder affecting an estimated 1 per 15,000-30,000 live births, with an increased disease frequency in some isolated and inbred populations [4–8]. In PCD, abnormal cilia/flagella motility leads to a number of symptoms. Ineffective mucociliary clearance caused by respiratory epithelial cilia dysmotility gives rise to chronic, destructive upper and lower airway disease manifesting with recurrent respiratory infections, chronic sinusitis and otitis media, usually evident from the first year of life and progressing to permanent lung damage (bronchiectasis) [4, 9]. Individuals affected by PCD are often subfertile, occasionally manifest hydrocephalus, and their left-right axis determination is randomized with about half having situs abnormalities (Kartagener syndrome [combined PCD and situs inversus] [MIM 270100]) resulting from embryonic node cilia dysfunction during development [10, 11]. This causes complex malformations in ~6% of cases, often associated with congenital heart disease [12–14].

PCD is genetically heterogeneous and associated with a variety of axonemal ultrastructural defects. Mutations causing PCD have been defined in 17 genes, in addition to *RPGR* (MIM 312610), which causes syndromic disease [15]. Loss of the outer dynein arms is the commonest ciliary defect observed in PCD (>65% of cases), caused by mutations in ODA components (*DNAH5* [MIM 603335], *DNAI1* [MIM 6043661], *DNAI2* [MIM 605483], *DNAL1* [MIM 602135], *TXNDC3* [MIM 607421] or in genes encoding proteins involved in ODA assembly and stability causing accompanying inner dynein arm defects (*LRRC50/DNAAF1* [MIM 613190], *KTU/DNAAF2* [MIM 612517], *DNAAF3* [MIM 614566], *CCDC103* [MIM 614677], *HEATR2* [MIM 614864], *LRRC6* [MIM 614930]) [3, 16–26]. An exception is *DNAH11* (MIM 603339), which encodes an ODA protein but is associated with a normal ultrastructure [27,

28]. Mutations have also been reported in radial spoke genes (*RSPH4A* [MIM 612647] and *RSPH9* [MIM 612648]), nexin-dynein regulatory complex genes (*CCDC39* [MIM 613798] and *CCDC40* [MIM 613799]) and central pair apparatus genes (*HYDIN* [MIM 610812]) [29–32].

Here, we first sought to identify the genetic defect in PCD-affected families from Volendam, a single fishing village in North Holland that has been genetically isolated for geographic and religious reasons since the 15th century [33]. This genetic bottleneck effect has increased by 50 or 100-fold the risk of PCD to at least 1 per 400, as shown by the fact that we have recorded >56 individuals (among the current population of approximately 22,000) affected by PCD in Volendam who are registered at family physicians. The carrier frequency of the mutation in this population can thus be estimated at 1 in 10. For genetic studies, signed and informed consent was obtained from all participants according to protocols approved by the institutional ethics review boards. We used genomic DNA isolated from peripheral blood samples from a total of eight Volendam families. PCD-01 is a large multigeneration family with 8 affected individuals which was shown from genealogical studies using available church records to originate from three ancestral marriages, with extensive inbreeding throughout the subsequent generations (figure 1A). The seven other families included 8 affected individuals (PCD-02 to PCD-08, figure 1A). These families were not aware of immediate blood connections to each other, but surnames were shared among the family of PCD-01 III:8 and three of the smaller families, suggesting that historical relationships do exist.

All 16 individuals affected with PCD from Volendam share a similar disease course including typical PCD symptoms of early neonatal respiratory symptoms (cough, increased mucus production and shortness of breath), pneumonia, and/or atelectasis (partial lung collapse) (table 1). During the course of disease, these individuals variously manifested with otitis media, chronic respiratory infections, chronic cough and pneumonia. This induces haemoptysis and requires hospital visits because of infections with a variety of pathogens, including *Pseudomonas aeruginosa*. Six affected individuals from Volendam (38%) have situs-related abnormalities, either complete left-right organ reversal or isolated thoracic/abdominal complications, with complex heart malformations in two cases (table 1). Where information is available, all affected individuals had documented bronchiectasis or the early signs of it (table 1, figure 1B). The high disease incidence in Volendam is intriguing because infertility is often associated with PCD. It is therefore notable that five affected individuals from Volendam had children, with offspring that included affected individuals in two cases (PCD-01 II:4 and II:7) (table 1). Fertility problems were not reported by any Volendam families. One male affected individual homozygous for the mutation with children underwent fertility testing in the past, but it showed a normal sperm count and motility; paternity was confirmed by marker analysis (Powerplex system, Promega).

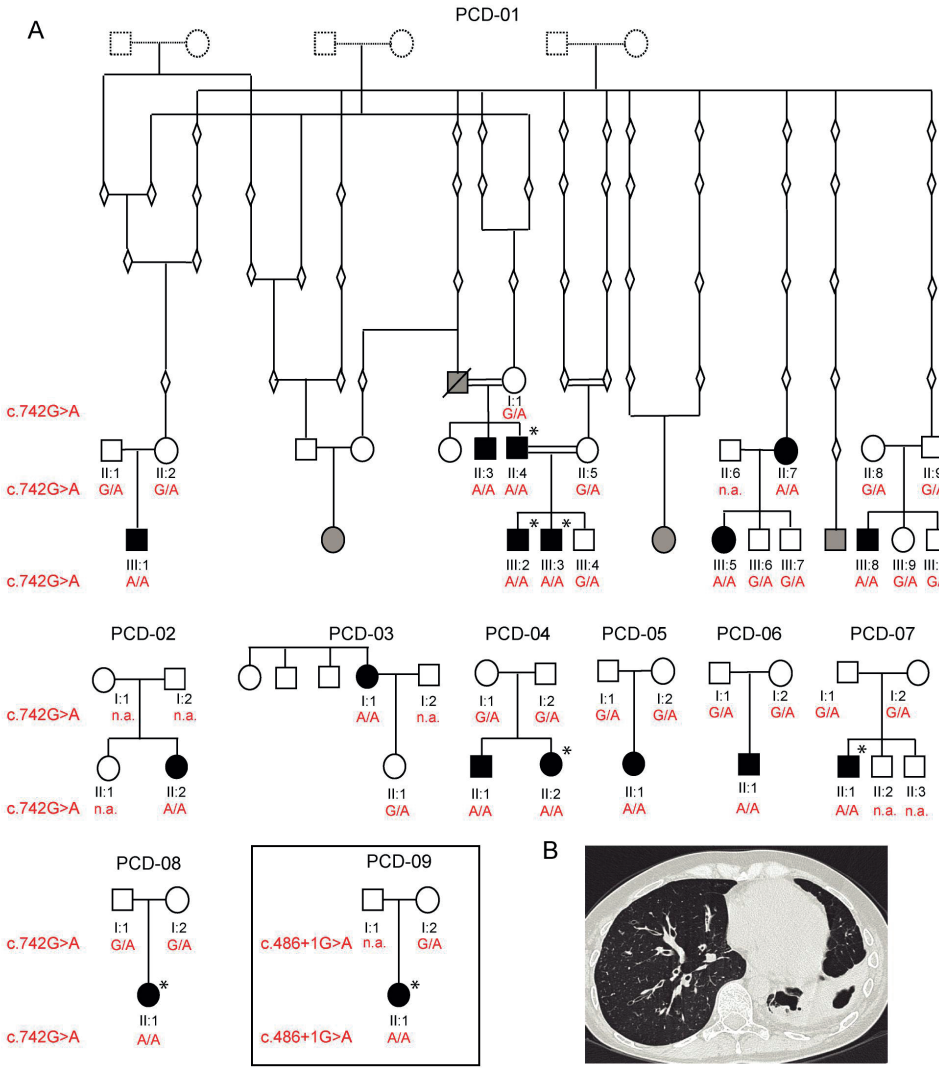

Figure 1. Segregation analysis of CCDC114 mutations

A. Pedigree structure of Volendam families PCD-01 – PCD-08 showing the segregation of the c.742G>A mutation and of UK family PCD-09 (boxed) showing segregation of the c.486+1G>A mutation. The genealogy of PCD-01 is derived from available church records. Not all ascertained individuals have been shown in the pedigrees, for reasons of space. Filled symbols indicate affected individuals, clear symbols indicate unaffected individuals, grey indicates affected individuals for whom samples could not be obtained, diamonds and dashed symbols indicate confirmed older individuals where samples are unavailable. Asterisks indicate situs abnormalities were reported. B. High resolution computed tomography (HRCT) chest scan of an affected Volendam individual showing bronchiectasis of the right and left lower lobe of the lung.

Table 1. Clinical phenotype of the PCD cases from the Volendam and UK population

ID	Cilia dysmotility	Neonatal symptoms	Situs	CHD	Chronic wet cough	Serous Otitis Media	Sinusitis	BX on CT	Chronic abnormalities on CXR	Lobectomy	Hemoptysis	Recurrent bacterial presence	Recurrent pneumonia	*P. aeruginosa*	Fertility defect
PCD-01 II:3	n.a.	Dyspnoea, feeding problems	Situs solitus	N	Y	Y	Y	Y	Y	N	n.a.	Y	N	n.a.	n.a.
PCD-01 II:4	n.a.	n.a.	Isolated dextrocardia	N	Y	N	Y	Y	Y	N	N	Y	Y	N	N; 3 children
PCD-01 II:7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	N; 3 children
PCD-01 III:1	n.a.	RDS, atelectasis	Situs solitus	Y[a]	Y	Y	Y	n.a.	n.a.	N	Y	No cultures obtained	N	No cultures obtained	n.a.
PCD-01 III:2	n.a.	Dyspnoea	Situs inversus totalis	N	N	Y	Y	n.a.	n.a.	N	Y	No cultures obtained	N	No cultures obtained	Untested, too young
PCD-01 III:3	n.a.	N	Situs inversus totalis	N	Y	Y	N	n.a.	n.a.	N	N	No cultures obtained	N	No cultures obtained	Untested, too young
PCD-01 III:5	n.a.	Wet cough	Situs solitus	N	Y	Y	N	Y	Y	LLL	Y	Y	Y	Y	N; 2 children
PCD-01 III:8	n.a.	Pneumonia	Situs solitus	N	Y	Y	N	n.a.	n.a.	N	N	N	N	N	N
PCD-02 II:2	Y	Pneumonia	Situs solitus	N	Y	Y	Y	Y	Y, signs of BX	MRL, LLL	Y	Y	Y	N	N; 2 children
PCD-03 I:1	n.a.	n.a.	Situs solitus	N	Y	Y	Y	Y	Y	N	N	Y	N	Yes, recurrent	N; 2 children
PCD-04 II:1	Y	Pneumonia	Situs solitus	N	Y	Y	N	n.a.	Y, signs of BX	N	N	Y	N	Y, 1x 2007, cleared	Untested, too young
PCD-04 II:2	Y	Pneumonia	Abdominal situs inversus	N	Y	Y	N	n.a.	Y, signs of BX	N	N	Y	N	N	Untested, too young
PCD-05 II:1	Y	Wet cough	Situs solitus	N	Y	Y	n.a.	n.a.	Y, signs of BX	N	N	Y	Y	Y, 1x 2010, cleared	Untested, too young
PCD-06 II:1	Y	Rhinorrhoea	Situs solitus	N	Y	Y	Y	n.a.	Y, recurrent atelectasis, wall thickening	N	Y	Y	N	Y, 1x 2006, cleared	Untested, too young
PCD-07 II:1	Y	Sputum during feedings	Situs inversus totalis with medial heart position	Y[b]	Y	Y	N	n.a.	Y, moderate wall thickening	N	N	Y	Y	N	Untested, too young
PCD-08 II:1	n.a.	RDS, atelectasis	Situs inversus totalis	N	Y	N	N	n.a.	n.a.	N	N	N	N	N	Untested, too young
PCD-09 II:1	Y	Dyspnoea	Situs inversus totalis	Y	n.a.	Y	Y	Y	Y	Y	n.a.	Y	n.a.	Y	N; has children

Abbreviations: RDS, respiratory distress syndrome; CHD, congenital heart disease; BX, bronchiectasis; CT, computed tomography; CXR, chest x-ray; LLL, left lower lobe; MRL, middle right lobe; *P.aeruginosa, Pseudomonas aeruginosa*; Fertility defect, i.e. ectopic pregnancy, unable to conceive, ever received in vitro fertilisation or intracytoplasmic sperm injection. [a]double outlet right ventricle, ventricular septal defect, aortic stenosis, persistent left vena cava superior, tricuspid valve insufficiency, pulmonary arterial hypertension; [b]atrial situs inversus with double discordance, pulmonary artery stenosis, ventricular septal defect.

We performed exome sequencing at the Wellcome Trust Sanger Institute (Cambridge, UK) as part of the UK10K project in two distantly related affected individuals from the extended Volendam pedigree: PCD-01 III:3 and PCD-01 III:8 (figure 1). Approximately 3µg of genomic DNA was sheared to 100-400bp by sonication (Covaris). Fragments were subjected to Illumina paired-end DNA library preparation and enriched for target sequences (Agilent SureSelect All Exon 50Mb kit) which were sequenced with 75bp paired-end reads on the HiSeq platform (Illumina). Sequencing reads that failed QC were removed with the Illumina GA Pipeline, and the rest were aligned to the reference human genome (GRCh37) by BWA (v0.5.9-r16). GATK (v1.1.5) was used to realign around known indels from the 1000 Genomes project and recalibrate base quality scores [34]. Alignments for a single sample were merged and duplicates marked. Variants were called per-sample using both SAMtools (v0.1.17) and GATK UnifiedGenotyper (v1.1.5), filtered on variant quality metrics separately, and the resulting data sets were merged. More than 6.60Gb of sequence was generated per sample, such that >77% of the target exome in both cases was present at greater than 20-fold coverage (table S2.1 available online).

Analysis of the exome variant profiles was performed using EVAR software tool vs 0.2.2 beta. We filtered variants for novelty by comparing them to 181 UK10K non-PCD exomes and by excluding those that were present in the 1000 Genomes Project polymorphism database with a minor allele frequency >0.005 [34]. Because the Volendam population is isolated and the PCD-01 III:3 individual is the offspring of a consanguineous marriage, we followed a model of rare autosomal-recessive inheritance. Therefore we focused on homozygous nonsynonymous and splice-site substitutions and indels that were shared by both members of the extended pedigree. This revealed *CCDC114* (RefSeq accession number NM_144577.3) as the only gene harbouring low-frequency variants meeting this criteria that were compatible with recessive inheritance (table S2.2).

CCDC114, located on chromosome 19q13.3, represented an excellent functional candidate, being the human gene orthologous to *Chlamydomonas DCC2*, which encodes an axonemal outer dynein arm microtubule-docking complex subunit [35]. Furthermore, in situ hybridization images of mouse embryos generated as part of the Eurexpress project and available within the Mouse Genome Informatics pages showed a strong pattern of gene expression in motile ciliated tissues, including the nasal cavity epithelium and brain ventricles [36]. Both affected Volendam individuals were homozygous for a c.742G>A substitution affecting the final G nucleotide of *CCDC114* exon 7, one of the consensus splice donor bases essential to the mRNA splicing machinery. This base change is therefore predicted to cause a frameshift in the CCDC114 protein resulting from loss of the conserved donor splice site. The c.742G>A substitution is also predicted to create a missense change p.Ala248Thr, but the p.Ala248 amino acid is not well conserved across species and the missense change

was predicted to be nondeleterious to protein structure according to programs that assess nonsynonymous SNPs (Polyphen-2, SIFT). We therefore concluded that the putative splicing defect predicted by this substitution was the more likely mutation mechanism.

The c.742G>A variant was confirmed by capillary sequencing (figure S2.1). Segregation analysis of the c.742G>A substitution in all available members of the PCD-01 pedigree confirmed the recessive inheritance of the variant (figure 1A). The same variant was then confirmed to segregate with disease in all available Volendam pedigrees, PCD-02 to PCD-08 (figure 1A). In total, all 16 affected Volendam individuals carry the c.742G>A variant as a homozygous change. This variant is reported in dbSNP v135 to be present at a very low frequency in heterozygous state in European descent controls from the NHLBI Exome Variant Server (rs147718607). The A allele is present at a frequency of less than 1 in 3,200 alleles, well below DNA polymorphism levels because the homozygous genotype would be extremely rare: this variant frequency would give a prevalence of homozygous cases of less than 1 in 40,000,000 (10^7).

We proceeded to sequence the *CCDC114* exons and flanking intronic regions in a larger cohort of 44 individuals affected with PCD resulting from ODA and combined ODA/IDA defects (primers listed in table S2.3). Signed and informed consent was obtained from all participants according to protocols approved by the institutional ethics review boards. Mutational analysis resulted in the identification of an additional homozygous splice site variant, c.486+1G>A, in one UK family, PCD-09 (figure S2.1). This substitution affects the *CCDC114* exon 5 consensus splice donor site, predicted to cause a frameshift in the CCDC114 protein product. This individual's disease was consistent with the Volendam cases, with typical features of PCD including bronchiectasis requiring lobectomy (table 1). The affected individual PCD-09 II:1 has situs inversus with congenital heart disease and had children with no reported fertility problems. Segregation analysis in PCD-09 family members confirmed a consistent recessive pattern of inheritance (figure 1A). The c.486+1G>A variant was absent from all the control sequence databases (dbSNP v135, 1000 Genomes Project, NHLBI Exome Variant Server).

We next used RNA isolated from ciliated cells of affected individuals and controls to assess the functional impact of the two *CCDC114* splice donor site mutations. Nasal brushings or curette biopsies were obtained from PCD-02 II:2 (c.742G>A homozygote) and PCD-09 II:1 (c.486+1G>A homozygote) and healthy volunteers. For PCD-02 II:2, the RNA was isolated from the cells after culture in standard conditions and for PCD-09 II:1 the RNA was isolated from noncultured cells [37]. RNA was extracted from the cells using TRIzol (Invitrogen) or the Quick RNA Miniprep Kit (Zymogen), and first-strand complementary DNA was synthesized using random nonamers (Sigma-Aldrich) or oligo-d(T)20 primer (Invitrogen) and Omniscript

transcriptase (Qiagen) or Superscript II reverse transcriptase (Invitrogen), respectively. PCR amplification was carried out using primers in exons 6 and 8 of *CCDC114* in PCD-02 II:2, and in exons 2 and 5 of *CCDC114* in PCD-09 II:1, in parallel to amplification of the same samples with the control housekeeping genes *GAPDH* (MIM 138400) and *ACTB* (MIM 102630), respectively. The RT-PCR primers are listed in table S2.4.

A larger RT-PCR product size was amplified from the PCD-02 II:2 c.742G>A Volendam individual, compared to controls (figure S2.2). Sequence analysis revealed an intronic insertion of 79 basepairs after the end of exon 7, shifting the protein reading frame and introducing a novel nonsense codon 52 residues downstream (p.Ala248Thrfs52*). Mutation prediction software (Alamut) suggests that this aberrant message is generated through utilisation of a cryptic splice donor site downstream of the mutated site, within intron 7 (figure 2A). Furthermore, incubation with 0.1 ml of a 50mg/ml ethanol solution of cycloheximide for 4.5 hours according to standard protocols to block protein translation indicated only a limited effect of nonsense-mediated decay on this mutation (figure S2.2). RT-PCR of the PCD-09 II:1 exon 5 c.486+1G>A UK individual's sample yielded no product despite repeated attempts to amplify, although the control had the expected RT-PCR product size; *ACTB* RT-PCR confirmed amplification of an equivalent product in both, suggesting that *CCDC114* gene expression is specifically disrupted (not shown). The aberrant mRNA in PCD-09 II:1 could be difficult to amplify if it contains a large intronic insertion comprising all or part of the large (5,229 bp) intron 5.

By qPCR we were able to investigate the effect of the c.742G>A mutation on *CCDC114* expression levels in the Volendam individual, PCD-02 II:2, by using a Roche Lightcycler to quantify mRNA in nasal epithelial cells cultured from PCD-02 II:2 compared to controls. The primers, probe design and method is described in table S2.5, with all gene expression normalized to the housekeeping gene *ACTB*. *CCDC114* ciliary transcript levels were significantly reduced in these actively ciliated cells compared to that of controls, but a low transcript level was retained in the affected individual (figure 2B). Thus, although the splicing defect severely abrogates gene expression, these data do not exclude the possibility of some remnant functional expression of *CCDC114* transcripts.

High speed video imaging of respiratory epithelial cells demonstrated the effect of CCDC114 deficiency on ciliary beat frequency, as well as amplitude and coordination of movement (Bassler A602F-2 camera and Image Pro software, and as previously described [38]). Both c.742G>A (movie S1) and c.486+1G>A (movie S3) epithelia compared to normal controls filmed in identical conditions (movies S2 and S4 respectively) showed abnormal ciliary motility comprising large areas of static cilia, with occasionally 1-2 cilia having a twitching or flickering movement that was stiff, slow and ineffective for mucus transport across the epithelial surface.

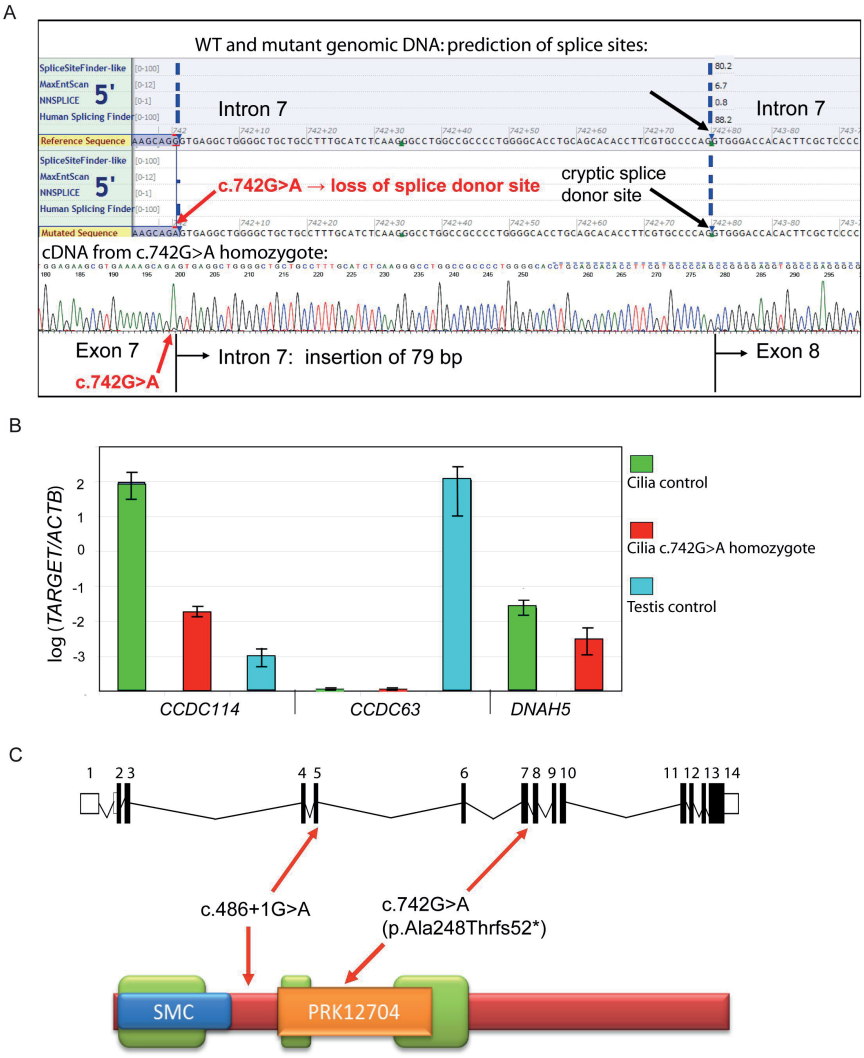

Figure 2. CCDC114 splice-site mutations causing primary ciliary dyskinesia

A. Effect of the c.742G>A Volendam mutation on splicing. The upper panels show the location of the mutation in genomic DNA sequence chromatograms, and the splice site prediction effect according to Alamut. Alamut uses the four different splice prediction software programs listed on the left. In comparison of the reference sequence from a control individual (top) against the mutant genomic DNA (bottoml), the software predicts loss of the splice donor site and presence of a cryptic splice site 79 bp into the intron. The bottom panel shows the sequence of cDNA from a person who is homozygous for the mutation, isolated from ciliary cells and amplified via primers in exons 6 and 8. An intronic insertion of 79 basepairs is present in the c.742G>A individual's cDNA, located between the mutation substitution site (green arrow) and the presumed intronic cryptic splice site (pink arrow). The sequence shows no indication of use of the regular splice donor site. The inclusion of 79 bases leads to a frameshift and a premature stop codon in exon 8 after addition of 52 novel amino acids, at the in-frame TAA codon indicated by the red box with arrow. B. Relative expression levels

(normalized to ACTB) of CCDC114, CCDC63 and DNAH5 in mRNA from testis and cultured nasal epithelial cells from controls or from Volendam PCD-02 II:2, assessed by qPCR using a Roche Lightcycler as described in Table S2.5. CCDC114 is expressed at higher levels in cilia-producing cells compared to testis whereas CCDC63 is expressed highly in testis with no detectable expression in cilia-producing cells. In addition, CCDC114 and DNAH5 levels are both reduced in cilia from the Volendam affected individual compared to control. The means ± SEM from triplicate repeat experiments are shown. C. Location of the Volendam and UK splice-site mutations in the intron-exon structure shown above, and in a model of the CCDC114 protein shown below. Black boxes indicate coding exons, white boxes noncoding exons. The green boxes indicate coiled-coil domains as detected by Paircoil2 run with a minimum window size of 28. Homology was also detected identifying an SMC (structural maintenance of chromosomes protein) domain in CCDC114 indicated by the blue box (SMC_prok_B TIGR02168), and a putative prokaryotic phosphodiesterase domain indicated by the orange box (PRK12704).

This is consistent with findings in other PCD individuals lacking the ODAs (with or without accompanying IDA loss) [39, 40]. Transmission electron microscopy of respiratory cilia cross-sections showed that all *CCDC114* mutant samples shared a common ciliary ultrastructural defect, a loss of the outer dynein arms (ODA) (figure 3A). This is consistent with EM findings in the *Chlamydomonas* strain *oda1* carrying null mutations in the *CCDC114* ortholog *DCC2* [41]. Interestingly, despite this lack of ODAs, the flagella of the *Chlamydomonas oda1* strain retain some ability to beat; however, they beat slowly and without the correct effective waveform [42]. This species difference when ODA components are deficient has been reported before [2].

The *Chlamydomonas* ortholog of CCDC114 (DCC2/ODA1) is a component of the ODA docking complex (ODA-DC) required for the assembly of ODAs onto the flagella peripheral doublet microtubules [35]. In *Chlamydomonas*, ODA-DCs are transported and assembled onto the peripheral microtubules independently from the ODAs that attach to them, and the ODAs cannot attach in their absence [43]. In *oda1* DCC2-null mutant strains, ODA-DCs are not assembled onto the axoneme's microtubules, and consequently neither are the ODAs [43]. We modelled the comparative protein structure of CCDC114 to investigate the potential functional impact of the identified splice site mutations. CCDC114, like *Chlamydomonas* DCC2, has three coiled-coil domains (figure 2C). Mutations in coiled-coil domain proteins are already associated with PCD, playing an important role in axonemal organization and cilia ultrastructure [24, 30, 31]. Coiled-coils were proposed as likely to be important for interactions between DCC2 and other docking complex subunits, and the domain between the second and third DCC2 coiled-coil domain was also proposed to participate in protein-protein interactions [35]. A conserved Structural Maintenance of Chromosomes (SMC) domain was also detected in CCDC114, similar to those identified to play a role in microtubule-based ciliary transport processes in the PCD-associated proteins CCDC39 and CCDC40 [30]. The c.742G>A and c.486+1G>A mutations would lead to the lack of either one or two critical coiled-coil domains, with apparently similarly deleterious consequences (figure 2C).

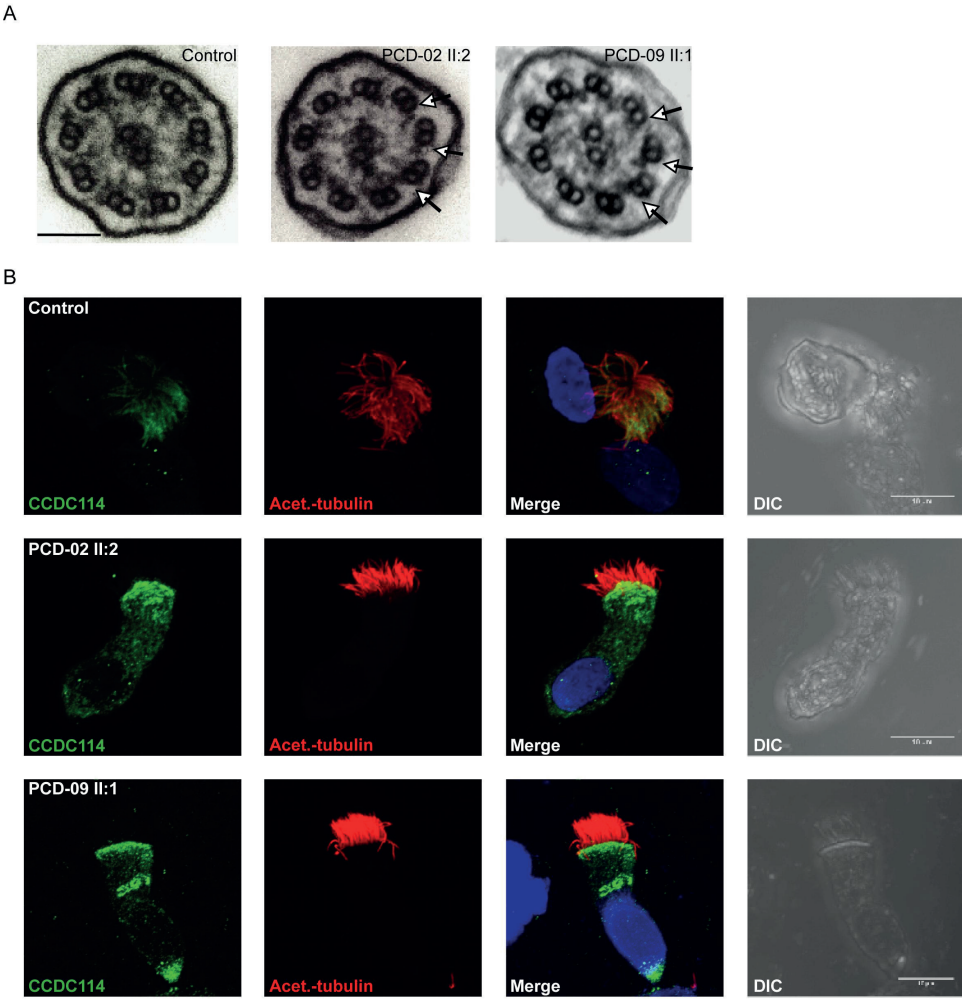

Figure 3. CCDC114 splice site mutations are associated with ciliary axoneme defects

A. Transmission electron micrographs of cross-sections of respiratory epithelial cell cilia demonstrates loss of outer dynein arms in both the PCD-02 II:2 and PCD-09 II:1 individuals carrying the c.742G>A and c.486+1G>A splice donor mutations, respectively. All nine peripheral doublets showed loss and reduction of the outer dynein arms (arrows) compared to controls. Scale bar, 100 nm. B. Subcellular localization of CCDC114 protein (green) in respiratory epithelial cells via a rabbit polyclonal antibody (Sigma HPA042524). In healthy individuals (top), CCDC114 is localized along the length of the axoneme of the ciliated cells, whereas in both PCD-02 II:2 and PCD-09 II:1, CCDC114 is markedly reduced (middle and bottom). Axoneme-specific anti-acetylated-α-tubulin antibody (Sigma) was used as a control to stain the entire axoneme (red). DNA (blue) was stained using DAPI (Invitrogen). Scale bars represent 10 µm.

To further investigate CCDC114 function, we analyzed protein localisation by high-resolution immunofluorescence microscopy in ciliated epithelial cells. In controls, CCDC114 antisera decorates the full length of the cilia (figure 3B), suggesting that its putative role in tethering of outer dynein arms is required along the entire axoneme. Two different classes of ODAs have been defined by Fliegauf et al. at the distal (DNAH5-positive, DNAH9-positive) and proximal (DNAH5-positive, DNAH9-negative) ends of cilia [40]. CCDC114 appears to be a component of ODA docking complexes capable of interacting with both ODA types. In contrast, the individuals carrying c.742G>A and c.486+1G>A mutations had severely reduced levels of CCDC114 along their entire cilia (figure 3B). By using well-established diagnostic markers of axoneme integrity developed by the Omran lab, we confirmed by staining with the ODA component DNAH5 that the ODAs along the cilia length are absent in c.742G>A or c.486+1G>A cells, whereas DNALI1 staining confirmed that the IDAs are present and undisturbed (figure S2.3, S2.4) [22]. It is not known whether human ODA-DCs and ODAs are transported by the same or different mechanisms to the axonemes, but in *Chlamydomonas* they can be assembled separately in the cytoplasm, and ODAs are assembled in the cytoplasm even without the ODA-DC being present [43]. However, the cells deficient for CCDC114 arising from either mutation did not show any noticeable cytoplasmic accumulation of ODAs by DNAH5 staining, indicating a possible species difference in these pathways (figures S2.3, S2.4). We investigated the expression levels of *DNAH5* mRNA by qPCR in cultured ciliated epithelial cells from the Volendam individual PCD-02 II:2 (Roche Lightcycler, table S2.5), normalizing to the housekeeping gene *ACTB*. There was a reduction in *DNAH5* levels in in PCD-02 II:2 compared to control, although this was less marked than the reduced *CCDC114* ciliary expression (figure 2B). These results are in agreement with the lowered CCDC114 protein expression seen via immunofluorescence; however, the lack of DNAH5 immunofluorescence may reflect enhanced degradation of the DNAH5 protein rather than a lack of its accumulation (figure 3 and S2.3).

Our data suggest a single ancestral *CCDC114* mutation c.742G>A underlies all Volendam PCD cases, most probably spread by genetic bottleneck founder effect. This village was founded in 1462 by 20 families who established a settlement after the nearby town of Edam dug a new exit to its sea harbour and dammed up the old exit. These families settled on the "filling dam" land or "Vollendam" and because of their isolated site, the church reformation in the late 16th century passed them without effect. The major religion remains Roman Catholic and even after their geographic isolation has lessened, their religious and social distinctions have kept the Volendam population very isolated into the modern era. A review of the unfiltered UK10 whole exome sequence data, to derive the available SNPs across the *CCDC114* locus, shows that the two distantly related individuals carrying the c.742G>A mutation share a 2 megabase haplotype (not shown). This supports the idea that the Volendam mutation was

spread within this inbred population from one original founding ancestor, and its small size explains why a single locus was missed in past linkage mapping. In the large PCD-01 pedigree (figure 1), common ancestors are found six to seven generations back, dating to the early 1800s. However, because not all the Volendam families could be connected and this small common haplotype was found to carry the mutation, presumably reduced by ancient meiotic recombination events, this suggests a more advanced age for the shared mutation than the founding of the Volendam village. According to the genetic maps of Genethon, Marshfield and DeCode, this 2 Mb region on chromosome 19q13.33 spanning *CCDC114* corresponds to a genetic distance of 3.4-4.6 centiMorgan. Te Meerman et al. have shown that around a new mutation a mean haplotype sharing length of 5 cM is reached after ~70 generations [44]. This preceeds the founding of the village of Volendam, which occurred an estimated 22 generations ago. Consequently, most likely, two or more carriers with the *CCDC114* mutation were present among the original founding families of Volendam. The finding of four heterozygous European carriers in the NHLBI Exome Variant Server supports the hypothesis that this variant arose prior to the founding of Volendam and was brought in by original settlers.

We found that fertility was not greatly affected amongst individuals carrying *CCDC114* mutations. The reasons for this are not clear, but there may be some functional redundancy of CCDC114 in sperm. In *Chlamydomonas* the ODA-DC consists of the CCDC114 ortholog DCC2/ODA1 and two other proteins, DCC1/ODA3 and DLE3/ODA14 [35]. However, the human ODA-DC seems to be differently structured, because no definitive human homolog can be found for DCC1/ODA3 or DLE3/ODA14. Furthermore, there is a second human protein apart from CCDC114 with significant homology to DCC2: CCDC63, which is 26% identical to CCDC114. CCDC63 is also 21% identical to the *Chlamydomonas* ODA5 protein that is associated with the axoneme and is required for outer dynein arm assembly but independent from the ODAs and ODA-DCs [45]. Whether CCDC63 plays an orthologous role to DCC2/CCDC114 or to ODA5 is not yet clear, but *CCDC63* represents an excellent candidate gene for an overlapping phenotype to that associated with *CCDC114* mutations [2, 46]. The relative levels of CCDC114 and CCDC63 proteins in the axoneme of sperm is not well understood, but available evidence from public expression databases such as Unigene suggests that the *CCDC63* transcript is more highly sperm-specific in its expression than *CCDC114*, and thus it is not impossible that CCDC114 function could be partially replaced by CCDC63 in sperm.

To test this hypothesis we used qPCR (table S2.5) on mRNA from testis (Life Technologies), the source of sperm cells (used because in sperm there is no active transcription), and from cultured nasal epithelial cells that were actively producing cilia. The nasal cells were derived both from controls and also the Volendam individual, PCD-02 II:2. A high expression

of *CCDC63* was detected in control testis, with no detectable expression in control cilia-producing cells, even after adding 10 cycles to the qPCR, whereas *CCDC114* is expressed at >100 times higher levels in cilia-producing cells compared to testis (figure 2B). Without a testis biopsy from an affected person we cannot exclude the possibility that affected individuals could retain some testis expression of *CCDC114;* however we can conclude that the level of *CCDC63* expression in control testis is comparable to *CCDC114* expression in control ciliary cells, and >100 times higher than *CCDC114* in testis.

In summary, we report mutations within conserved *CCDC114* splice donor sites affecting a total of 17 individuals with PCD, all homozygous for either c.742G>A or c.486+1G>A substitutions, conferring PCD with outer dynein arm loss, cilia immotility and laterality defects including complex cardiac malformations. Recent large-scale studies show the importance of this phenotype, estimating that 65-67% of PCD cases have outer dynein arm deficiencies, either alone (33-43%) or with other structures involved [47, 48]. We reveal that CCDC114 has a highly conserved role in ODA microtubular attachment, with a likely role as an integral protein of the ODA-DC, the loss of which prevents ODAs from binding onto axonemal microtubules. We identified a difference in relative expression levels of *CCDC114* that might suggest it has a more prominent role in cilia compared to testis. Identification of the Volendam founder mutation c.742G>A highlights CCDC114 as an important target for future therapeutic intervention, particularly in this at-risk population that has a high prevalence of PCD.

SUPPLEMENTAL DATA

Supplemental data include the UK10K Consortium author list, four figures, five tables and four movies and can be found with this article online at http://www.cell.com/AJHG/.

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WEB RESOURCES

The URLs for data presented herein are as follows:
Mouse Genome Informatics, http://www.informatics.jax.org/
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
1000 Genomes Project, http://www.1000genomes.org
OMIM, http://www. omim.org
SMART, smart.embl-heidelberg.de/
CDD, http://www.ncbi.nlm.nih.gov/sites/entrez?db=cdd
Paircoil2, groups.csail.mit.edu/cb/paircoil2/
PSI-BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi
STRING 9.0, string-db.org/
NHLBI Exome Variant Server/Sequencing Project (ESP), http://evs.gs.washington.edu/EVS/
UK10K Consortium, http://www.uk10k.org/

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3

MUTATIONS IN *PIH1D3* CAUSE X-LINKED PRIMARY CILIARY DYSKINESIA WITH OUTER AND INNER DYNEIN ARM DEFECTS

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ABSTRACT

Defects in motile cilia and sperm flagella cause primary ciliary dyskinesia (PCD), characterized by chronic airway disease, infertility and left-right body axis disturbance. Here we report maternally inherited and *de novo* mutations in *PIH1D3* in four men affected with PCD. *PIH1D3* is located on the X chromosome and is involved in the preassembly of both outer (ODA) and inner (IDA) dynein arms of cilia and sperm flagella. Loss-of-function mutations in *PIH1D3* lead to absent ODAs and reduced to absent IDAs, causing ciliary and flagellar immotility. Further, PIH1D3 interacts and co-precipitates with cytoplasmic ODA/IDA assembly factors DNAAF2 and DNAAF4. This result has clinical and genetic counseling implications for genetically unsolved male case subjects with a classic PCD phenotype that lack additional phenotypes such as intellectual disability or retinitis pigmentosa.

Motile cilia and flagella are microtuble-based organelles that extend from the surface of many cell types in the human body. Function of both flagella and cilia requires well-coordinated ATP-dependent interactions between the peripheral microtubuli and the axoneme [1]. The basic structure of motile cilia consists of a ring of nine peripheral microtubule doublets surrounding one central pair (9+2 structure). The peripheral ring is connected to the central pair (CP) through radial spokes (RSs) and the nexin-dynein regulatory complex (N-DRC) connects adjacent doublets. The CP, N-DRC and inner dynein arms (IDAs) are responsible for modulation and regulation of the ciliary movement while the outer dynein arms (ODAs) are responsible for beat generation [2–5]. ODAs and IDAs are large multimeric protein complexes that are pre-assembled in the cytoplasm before transported to the axonemes [6, 7]. The identification of proteins responsible for correct assembly, DNAAFs, and the composition of these protein complexes are critical to understand the patho-mechanisms of motile cilia-related diseases such as primary ciliary dyskinesia (PCD).

Primary ciliary dyskinesia (MIM 244400) affects an estimated 1:15,000 live births and is characterized by abnormal ciliary and flagellar movement, leading to numerous severe health issues [8]. Ineffective mucociliary clearance causes mucus stasis in the entire respiratory tract, leading to recurrent infections and chronic inflammation. Dysfunctional cilia at the embryonic node give rise to laterality defects such as situs inversus of the internal organs in about half of the individuals [9]. In addition, PCD-affected individuals often suffer from sub –or infertility caused by dysfunction of fallopian tube cilia and sperm flagella.

PCD is a genetically heterogeneous ciliopathy owing to the large number of proteins that are involved in ciliary motility. So far, autosomal recessive mutations in 30 genes account for an estimated 70% of cases [10]. Genetic analyses of PCD-affected individuals identified several autosomal recessive mutations in genes encoding for axonemal subunits of the ODA and ODA-docking complexes (ODA-DCs) [11–25]. In addition, recessive mutations in genes encoding other components of the ciliary motility apparatus as well as proteins required for motile ciliogenesis have been identified to be disease causing. An overview of these genes is given by Werner and colleagues [26]. Among those are also genes encoding proteins involved in cytoplasmic pre-assembly of ODAs and IDAs that have emerged from mutation analyses of PCD-affected individuals: *DNAAF1* (*LRRC50*, [MIM 613190]) [27, 28], *DNAAF2* (*KTU*, [MIM 612517]) [29], *DNAAF3* (*C19orf51*, MIM 614566)[30], *DNAAF4* (*DYX1C1*, MIM 608709)[31], *DNAAF5* (*HEATR2*, MIM 614864)[32], *LRRC6* ([MIM 614930]) [33], *ZMYND10* (MIM 607070) [34, 35], *SPAG1* (MIM 603395) [36] and *C21ORF59* (MIM 615494) [37]. Additionally, two X-linked PCD variants have been reported and are associated with syndromic cognitive dysfunction or retinal degeneration caused by mutations in *OFD1* (MIM 311200) and *RPGR* (MIM 312610), respectively [38, 39]. Here, we describe an example of X-linked non-syndromic PCD caused by mutations in *PIH1D3*.

We assembled a cohort of 75 PCD-affected individuals. Diagnosis of PCD was based on a combination of clinical symptoms and examination of ciliary motion by high-speed videomicroscopy (HSVM), ciliary ultratructure by transmission electron microscopy (TEM), and nasal nitric oxide production rate. We excluded individuals from the Volendam population carrying a previously identified *CCDC114* founder mutation [22].

We screened these 75 PCD-affected individuals with a targeted-exome panel of 310 genes. This panel included 26 PCD-associated genes and a subset of candidate genes. These candidate genes were selected based on (1) at least 10-fold higher gene expression via RNA microarray (data not shown) during *in vitro* ciliogenesis of human airway epithelium cells, and (2) a previous association to ciliary or flagellar proteins or processes in published candidate gene lists and the Gene Network database. Signed and informed consent was obtained from all individuals fulfilling the diagnostic criteria of PCD and their family members, according to protocols approved by the Institutional Ethics Review board at the VU University Medical Center (Amsterdam, The Netherlands) and by the Institutional Ethics Review board at the University of Muenster (Muenster, Germany). Targeted-exome sequencing of genomic DNA was performed at the VU University Medical Center and at the Cologne Center for Genomics (CCG). For enrichment, the Truseq DNA LT Sample prep kit or the NimbleGen SeqCap EZ Human Exome Library v2.0 was used. Enriched preparations were sequenced with the HiSeq2000 platform or the HiSeq2500 (Illumina) as paired end 2 x 100 base pairs reads. The 30x coverage reached 92.4%. Sequencing reads that passed quality filtering were mapped to the reference genome sequence (hg19). Variants that were present in dbSNP, the 1000 Genomes Project and Exome Aggregation Consortium (ExAC) with a minor allele frequency >0.01 were excluded. We focused on nonsynonymous mutations, splice-site substitutions and indels following an autosomal-recessive and X-linked inheritance pattern.

This approach revealed a hemizygous mutation in *PIH1D3* (Genbank: NM_001169154.1, c.357_363del [p.Val120Leufs*6]) in PCD-10 II-1 (figure 1A) and a hemizygous nonsense mutation (c.355C>T [p.Gln119*]) within exon 6 of *PIH1D3* OP-1899 II-1 (figure 1A). Neither mutation is reported in genomic-variant databases such as dbSNP, 1000 genomes, the Exome Variant Server (EVS) or the ExAC database. Both affected individuals show classical PCD symptoms such as chronic sinusitis, chronic otitis media, and chronic lower respiratory tract infections as well as bronchiectasis in the middle lobe and mucus plugging (shown for OP-1899 II-1 in figure 1B). In addition, both have situs inversus totalis (shown for OP-1899 II-1 in figure 1B) and had neonatal respiratory distress syndrome, a typical finding often present in PCD.

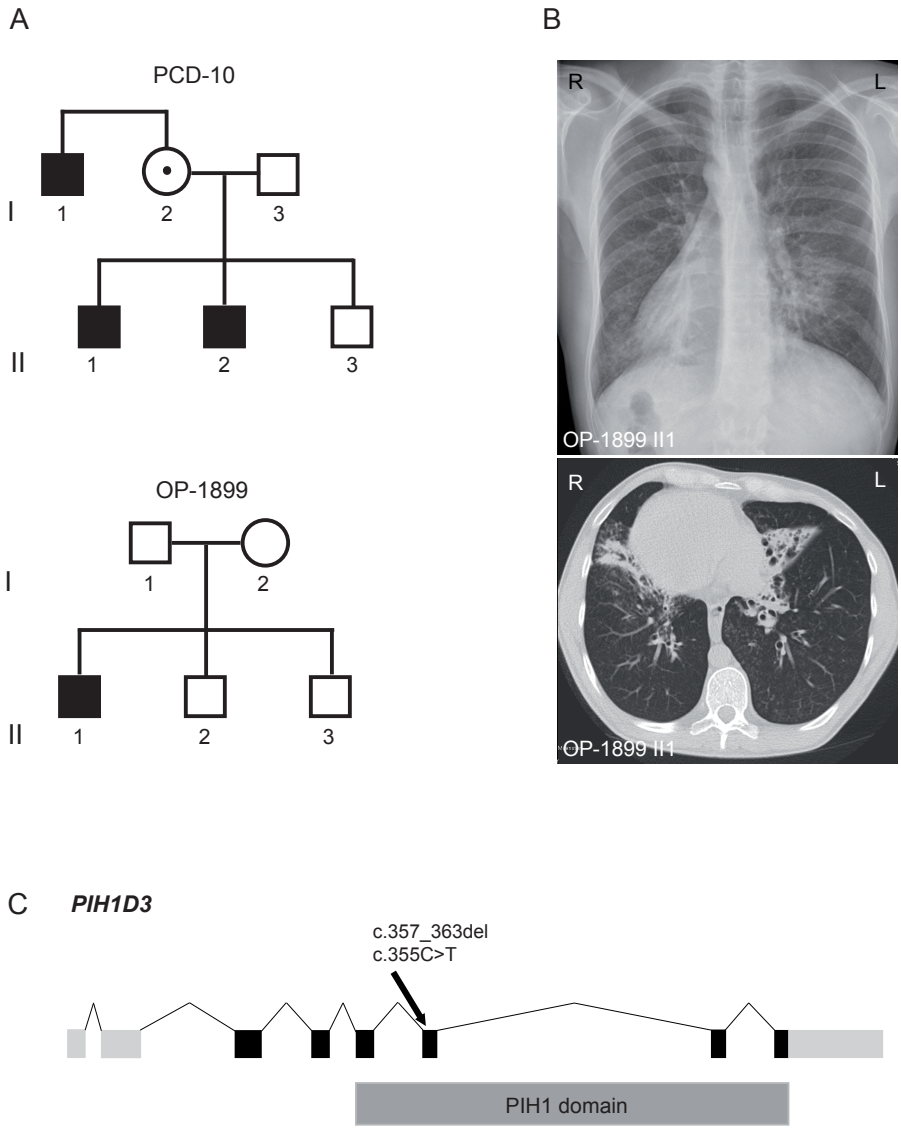

Figure 1. Results of the mutational analyses in PIH1D3, pedigrees of the PCD-affected families PCD-10 and OP-1899 and clinical features of OP-1899 II1.

(A) Hemizygous loss-of-function mutations in PIH1D3 located on the X chromosome were identified in two families. (B) Pedigree of PCD-affected families PCD-10 and OP-1899. PCD-affected siblings are shaded black and the unaffected sibling is shaded white. The chest X-ray radiograph as well as two computed tomography scans show situs inversus totalis, chronic airway disease with bronchiectasis in the middle lobe, and mucus plugging in OP-1899 II-1. (C) Exon-intron structure of PIH1D3. The exon-intron structure of PIH1D3 with untranslated (light grey) and translated regions (black) and the PIH1 domain (dark gray).

Table 1. Diagnostic characteristics

Affected individuals	TEM defect	HSVM	nNO [nL/min]	Nasal congestion	Neonatal period	SI	Chronic otitis media	Recurrent respiratory infections	Chronic wet cough	Fertility defect
PCD-10 I:1	ODA	Immotile	2	Yes	Unknown	No	Yes	Yes	Yes	Yes, 2 adopted children
PCD-10 II:1	Not enough cilia	Immotile	5	Yes	Pneumonia	Yes	Yes	Yes	Yes	Yes
PCD-10 II:2	ODA	Immotile	4	Yes	Pneumonia	No	Yes	Yes	Yes	Yes
OP-1899 II1	ODA	Immotile	38	Yes	Respiratory distress syndrome	Yes	Yes	Yes	Yes	Yes

Abbreviations: EM, electron microscopy; HSVM, high-speed videomicroscopy; nNO, nasal nitric oxide; SI, situs inversus; ODA, outer dynein arm.

Nasal nitric oxide measurement, high-speed videomicroscopy in sequential monolayer-suspension cell culture and transmission electron microscopy (TEM) was performed on all individuals of the PCD-10 family as previously described [22]. Nasal NO production rate was measured either by using a hand-held Niox Mino (Aerocrine) or EcoMedics CLD88 (Duernten) and was low in all affected individuals (table 1). Careful analysis of cilia from PCD-10 I-1, PCD-10 II-1 and PCD-10 II-2 and OP-1899 II-1 showed complete immotile respiratory cilia (movie S1 and S2) when compared to controls (movie S3 and S4). OP-1899 II-1 showed complete immotile sperm flagella (movie S5) when compared to control subjects (movie S6). The motility findings in respiratory cilia and sperm flagella are consistent with a loss of ODAs. Additionally, PCD-10 II-1 and PCD-10 II-2 had undergone fertility testing in the past, which showed largely immotile sperm flagella. TEM analyses of respiratory cilia of all affected showed absence of ODAs and a possible reduction of IDAs (figure 2A).

To explore the possibility of an X-linked recessive inheritance mode in both families, we investigated the pedigrees (figure 1A). Individual PCD-10 II-1 has a brother (PCD-10 II-2) and uncle (PCD-10 I-1) with similar respiratory symptoms and a history of sub– or infertility (table 1). Sanger sequencing confirmed the mutation in PCD-10 I-1, PCD-10 II-1 and PCD-10 II-2 and in one of the alleles of the mother of individuals PCD-10 II-1 and PCD-10 II-2, confirming an X-linked recessive inheritance mode (figure 1A). Pedigree analysis of family OP-1899 shows that OP-1899 II-1 has two unaffected brothers who did not carry the hemizygous mutation. Segregation analyses in family OP-1899 revealed that this is a de novo mutation. We sequenced *PIH1D3* in an additional 40 male PCD-affected individuals with ODA or combined ODA/IDA defects for mutations in *PIH1D3* but did not identify additional mutations.

PIH1D3 (also known as *CXorf41*) is located on chromosome Xq22.3, comprises 8 exons and, encompasses approximately 38 kb of genomic DNA. PIH1D3 is one of 4 members of a protein family interacting with Hsp90 (PIH1). Studies in *Chlamydomonas* suggest that PIH1 functions in the preassembly of axonemal dyneins but may also function in preribosomal RNA processing [41, 42]. Mutations in *DNAAF2/KTU*, also containing a PIH1 domain, cause PCD by interferring with cytoplasmic preassembly of both ODAs and IDAs in respiratory cilia and sperm flagella [29]. Further, *PIH1D3* mouse and zebrafish orthologues have previously been linked to flagellar motility defects and polycystic kidney disease, respectively [42, 43]. *Pih1d3*$^{-/-}$ mice almost completely lack sperm ODAs and show a reduced number of IDAs. The mutations we identified in both families lie in the crucial PIH1 domain (figure 1C) and are expected to cause either a truncated protein or no protein at all as a consequence of nonsense-mediated mRNA decay (NMD).

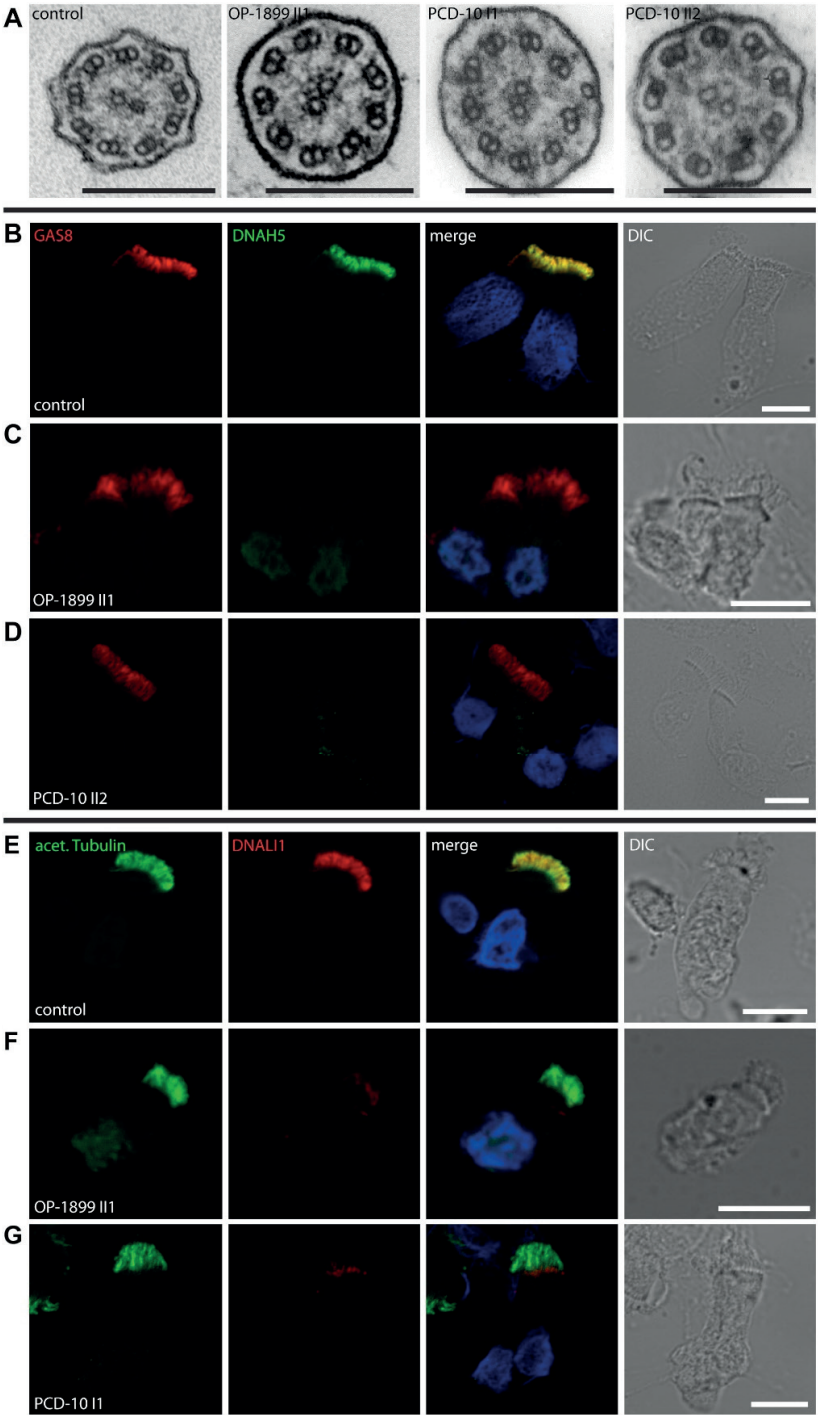
A
control
OP-1899 II1
PCD-10 I1
PCD-10 II2
B
GAS8
DNAH5
merge
DIC
control
C
OP-1899 II1
D
PCD-10 II2
E
acet. Tubulin
DNALI1
merge
DIC
control
F
OP-1899 II1
G
PCD-10 I1

Figure 2. Loss-of-function PIH1D3 mutations result in absence of outer and inner dynein arms in respiratory epithelial cells.

(A) The analysis of transmission electron microscopy cross-sections from respiratory cilia from OP-1899 II-1, PCD-10 I-1 and PCD-10 II-2 showed a normal 9+2 architecture with absence of outer dynein and inner dynein arms. Scale bars represent 200 nm. (B-D) Respiratory epithelial cells from control (B) and PCD-affected individuals OP-1899 II-1 (C) and PCD-10 II-2 (D) were double-labeled with antibodies directed against outer dynein arm heavy chain DNAH5 (green) and the N-DRC component GAS8 (red). Both proteins colocalize along the cilia in cells from the unaffected controls (B, yellow). In contrast, in mutant cells, DNAH5 was absent from or severely reduced in ciliary axonemes (C and D). The green staining in the nuclei observed in (C) is most probably background. (E-G) Cells were double-labeled with antibodies directed against acetylated tubulin (green) and the inner dynein arm light chain DNALI1 (red). In unaffected control cilia, both proteins colocalize along the ciliary axonemes (E, yellow). In contrast, in cells of individuals carrying PIH1D3 mutations, DNALI1 was absent from or severely reduced in the ciliary axonemes (F and G). The red staining at the ciliary base is an artefact caused by the polyclonal rabbit antibody [40] Nuclei were stained with Hoechst33342 (blue). Scale bars represent 10 µm.

To assess the functional impact of the c.357_363del mutation, we synthesized cDNA from isolated RNA of respiratory ciliated cells of PCD-10 I-1, PCD-10 II-1 and PCD-10 II-2 and a healthy control subject. RNA was isolated after sequential monolayer-suspension cell culture, as described previously [22]. PCR amplification of cDNA was carried out with primers for exons 5 and 7 of *PIH1D3*. PCR amplified one transcript in wild-type cells and two transcripts in mutant cells (figure S3.1A). Sequence analysis of mutant mRNA revealed a transcript that includes the c.357_363del in exon 6 and an alternative splice product that skips exon 6. Each of these transcripts alters the reading frame to introduce a premature termination in exon 6 and exon 8, respectively, resulting in truncated proteins of 125 amino acids (c.357_363del) and 181 amino acids (alternative splice product), respectively. This is in contrast to the 214 amino acid full-length protein.

To determine whether NMD degrades these transcripts, we incubated respiratory cells of individual PCD-10 I-1 with 0.25 mg/ml cycloheximide for 4.5 hours to stabilize the RNA by blocking protein translation [22]. qPCR of cDNA from respiratory cells of PCD-10 I-1 with and without cycloheximide incubation showed a 76% reduced expression of *PIH1D3*, relative to house keeping gene *VCP*, in cells without cycloheximide (table S3.1). Sequence analysis (figure S3.1A) showed that while the two aberrant transcripts are present in equal proportion in cells witout cycloheximide incubation, the transcript containing the c.357_363del is produced predominantly in cells with cycloheximide incubation. The transcript that skips exon 6 is probably caused by loss of exonic splice enhance sites and produces p.Tyr112Leufs*32. This transcript terminates in the last exon, presumably rendering the transcript insensitive to NMD. The transcript containing the c.357_363del results in p.Val120Leufs*6 but is subject to NMD, as was shown in cDNA from cells cultured with or without cycloheximide (figure S3.1A). Both of these transcripts, if translated, will lead to severely altered proteins that are

functionally inactive. The c.355C>T mutation in individual OP-1899 II-1 introduces a stop codon just one triplet before the c.357_363del variant does and it is therefore expected to cause a similar effect.

Next we performed western blot analysis to investigate the effect of the c.357_363del mutation at the protein level. Nasal epithelial cells were lysed in NuPAGE LDS Sample Buffer with NuPAGE reducing agent to produce a whole cell lysate, which was subjected to gel electrophoresis in NuPAGE 4%–12% BT gels using the XCell SureLock electrophoresis system. Proteins were transferred to nitrocellulose membrane using the iBlot Dry Blotting system (Invitrogen) and blocked in Odyssey blocking buffer (Westburg). The membrane was incubated overnight in Odyssey blocking buffer with 0.1% Triton X-100 with primary antibodies against *PIH1D3* and actin. Details of the antibodies used are shown in table S3.2. Secondary antibody incubation was performed for 1 hr with the IRDye 800 CW goat anti-rabbit IgG and the IRDye 680 CW goat anti-mouse IgG antibodies. The Odyssey infrared imaging system equipped with the Odyssey v.4 software (LI-COR Biosciences) was used to vizualize fluorescence. Examination of respiratory epithelial cell lysates from individuals PCD-10 I-1, PCD-10 II-1 , PCD-10 II-2 failed to detect PIH1D3 in contrast to the healthy control (figure S3.1).

To further characterize the ultrastructural defect caused by loss of function of *PIH1D3,* we performed high-resolution immunofluorescence (IF) analyses of control and *PIH1D3* mutant respiratory cilia and sperm flagella as previously reported [12, 22]. High-resolution IF images were taken with a Zeiss Apotome Axiovert 200 or a Zeiss LSM880 and processed with AxioVision 4.8 or ZEN Black and Adobe CS4. Confirming the ODA defect previously observed by TEM, we found complete axonemal absence of the ODA heavy chains DNAH5 (figure 2) and DNAH9 in mutant respiratory cilia (figure S3.2) and complete loss of the ODA intermediate chains DNAI1 and DNAI2 in mutant respiratory cilia (figure S3.3). Thus, *PIH1D3* deficiency results in abnormal assembly of type 1 as well as type 2 ODA complexes [1, 44]. Additionally, DNAI1 and DNAI2 were both absent from mutant sperm flagella (figure 3), indicating that assembly of both ODA types is also disturbed in sperm cells, similar to findings in the *Pih1d3*$^{-/-}$ mice [42]. Interestingly, the inner dynein arm light chain DNALI1 was greatly reduced or absent in the respiratory ciliary axonemes (figure 2E-2G). These findings are consistent with a defective cytoplasmic preassembly of ODAs and IDAs. However, normal localization of GAS8 and CCDC114 along the mutant ciliary axonemes shows that the N-DRC and ODA-DCs are not affected by loss of function of PIH1D3 (figures 2 and S3.4). In contrast to our finding, respiratory cells are not disrupted in *Pih1d3*$^{-/-}$ mice. This can probably be explained by a species difference. Mice have two copies of *Pih1d3,* on the X chromosome and on chromosome 1, whereas *PIH1D3* in humans is located solely on the X chromosome.

The function of human *PIH1D3* has not been previously investigated and the precise function of mouse *Pih1d3* has likewise remained elusive. Dong et al. hypothesized that PIH1D3 may play a role in dynein arm stabilization as they detected endogenous protein-protein interaction between PIH1D3 and the chaperones HSP70 and HSP90 as well as DNAIC2 in mouse sperm. However, interaction with DNAAF 1,2, and 3 was not described [42].

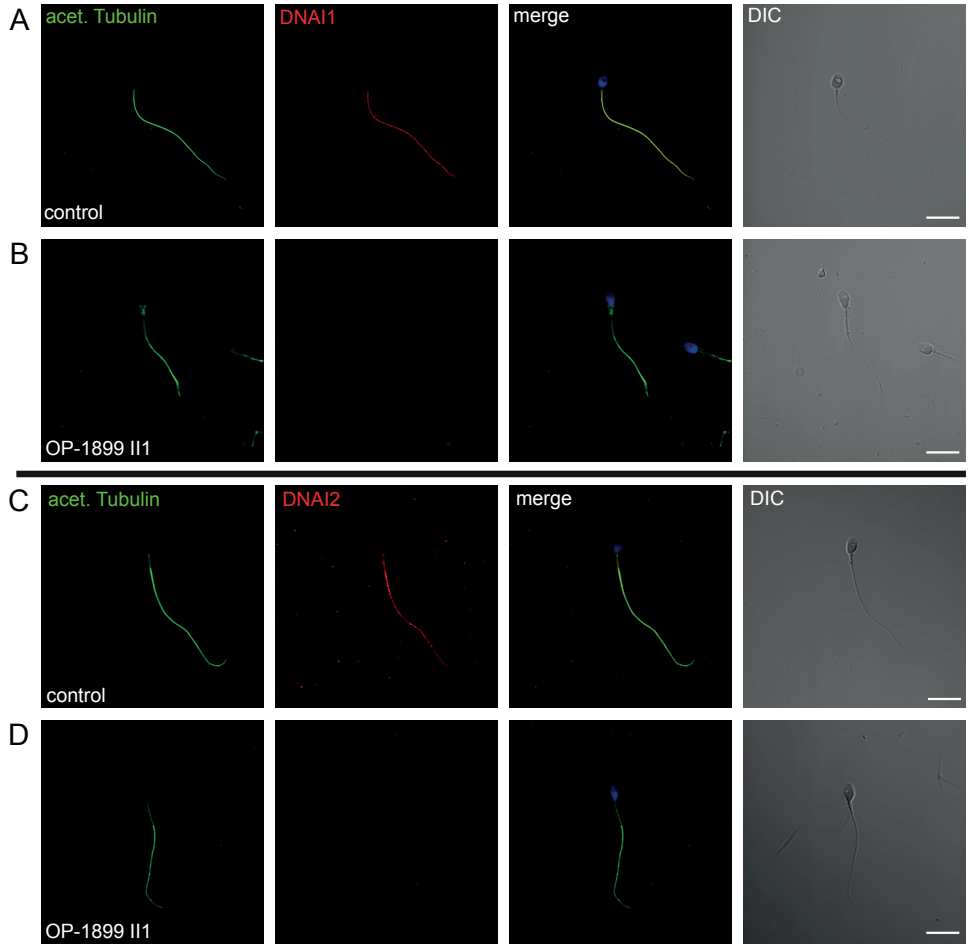

Figure 3. PIH1D3 mutant sperm flagella are deficient for the outer dynein arm intermediate chains DNAI1 and DNAI2.

Sperms were double-labeled with antibodies directed against acetylated tubulin (green) and DNAI1 or DNAI2 (both in red). DNAI1 and DNAI2 colocalize with acetylated tubulin along the flagellum in sperms from unaffected controls (yellow) (A and C). In contrast, in PIH1D3 mutant sperm tails, DNAI1 (B) and DNAI2 (D) were absent from flagellar axonemes. Nuclei were stained with Hoechst33342 (blue). Scale bars represent 10 µm.

In order to better understand *PIH1D3* function within this machinery, we performed a yeast two-hybrid (Y2H) screen using *PIH1D3* as a bait to test interaction with several genes previously associated with PCD, including dynein arm assembly factors (DNAAFs), genes possibly involved in dynein arm transport (*WDR69, IFT46*) and others. Direct interaction between *PIH1D3* and possible interactors was tested as previously described [31]. Human *PIH1D3* and *HSP90* clones were purchased from Origene. All other cDNAs have been previously cloned by nested PCR from human bronchial epithelial cell cDNA (ScienCell) using KOD polymerase according to manufacturer's directions as previously described [24]. Due to its large size (making it unsuitable for Y2H screening), *HSP90* cDNA was subcloned in fractions and similarly to all other cDNAs, recombined with pDONR201 Gateway vector via BP Clonase II reaction. Subsequently, cDNAs were subcloned into 3xHA and 3xFLAG epitope-tagged Gateway destination vectors via LR Clonase reaction. All cDNA clones were confirmed by sequence analysis and matched RefSeq gene accession numbers. *HSP90* was used as a positive control and all tested genes are shown in table S3.3. This led to the identification of two possible PIH1D3 interactors (figure 4A): DNAAF2/KTU and DNAAF4/DYX1C1. To confirm these interactions on the protein level, we performed co-immunoprecipitations using overexpression of tagged versions of the proteins in HEK293T cells. Whole-cell extracts and western blots were conducted as previously described [45]. Plasmids expressing C-terminal FLAG-tagged PIH1D3 were co-transfected with plasmids expressing C-terminal HA-tagged DNAAF2, DNAAF4, CCDC103 or p63 in HEK293T cells. At 24 hr after transfection, cells were lysed on ice in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40) supplemented with complete protease inhibitor cocktail (Roche Diagnostics). Lysates were incubated with either anti-FLAG M2-agarose from mouse (Sigma-Aldrich) or anti-HA affinity matrix (Roche) for 2–3 hr at 4 °C. After incubation, beads with bound protein complexes were washed in ice-cold lysis buffer. Subsequently, 4x NuPAGE sample buffer was added to the beads and heated for 10 min at 70 °C. Beads were precipitated by centrifugation, and supernatant was analyzed on NuPAGE Novex 4%–12% Bis-Tris SDS–PAGE gels. After blotting overnight at 4 °C, blots were stained with mouse anti-FLAG or mouse anti-HA. Fluorescence was analyzed on a LI-COR Odyssey 2.1 infrared scanner. Full unprocessed western blotting scans are shown in figure S3.5. PIH1D3 was consistantly able to precipitate DNAAF2/KTU and DNAAF4/DYX1C1 and vice versa (figure 4B). Our results using human cells confirm findings by Dong et al. for mouse sperm [42]. Similarly to the murine Pih1d3, human PIH1D3 interacts with the chaperone HSP90. In addition we detected protein-protein interactons between PIH1D3 and DNAAF2/KTU as well as DNAAF4/DYX1C1, indicating that PIH1D3 is possibly involved in dynein arm assembly and/or important for facilitating assembly enabled by these other DNAAFs. Interestingly, in contrast to *DNAAF2/KTU* or *DNAAF4/DYX1C1* deficiency that results in absence of ODA type-2, deficiency of *PIH1D3* results in absence of ODAs type 1 and type 2 [15, 22]. This observation indicates that the assembly of ODA type 1 is PIH1D3 dependent

and can occur independently from DNAAF2/KTU or DNAAF4/DYX1C1. Our results show an essential role for human PIH1D3 in the cytoplasmic preassembly process of both ODA types and IDAs, probably by stabilizing the formation of the DNAI1-DNAI2 complex.

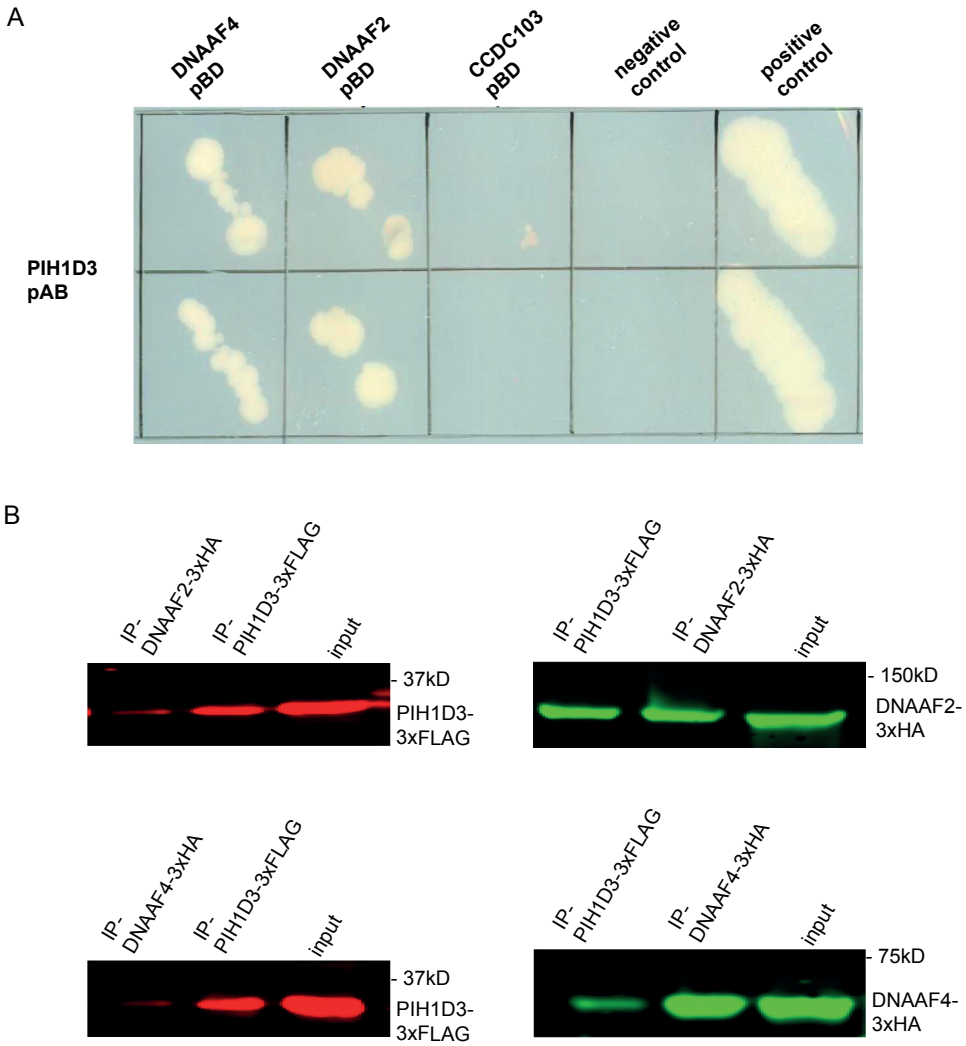

Figure 4. Identification of PIH1D3 interacting genes.

(A) Y2H assays show strong interaction of PIH1D3 with DNAAF2 and DNAAF4. Lamin-C-pBD - Gal4-pAD was used as a negative control and Gal4-pBD - Gal4-pAD as a positive control. (B) Confirmation of PIH1D3 interactors from the Y2H screen by co-IP from HEK293T cells. Co-expressions of tagged versions of PIH1D3 and either DNAAF2 or DNAAF4 revealed protein-protein interactions with DNAAF2 and DNAAF4 (left, western blot anti-Flag; right, western blot anti-HA).

Here we describe mutations in *PIH1D3*, a gene on the X chromosome, in PCD-affected individuals without coseggregation of severe intellectual disability or retinitis pigmentosa. This is of major clinical importance as symptoms related to primary ciliary dyskinesia were not the primary cause of diagnostic procedures undertaken in individuals described with pathogenic variants in cases presenting with mutations in the X-linked genes *RPGR* and *OFD1* [38, 39, 46]. Because mutations in other PCD-associated genes usually present with an autosomal-recessive mode of inheritance, it is possible that X-linked inheritance might be overlooked, especially in male cases (1) without syndromic coseggregation, (2) without knowledge of the entire pedigree, or (3) without siblings or with only female siblings. Additionally, loss of function of *PIH1D3* results also in sperm immotility and male infertility caused by disrupted assembly of ODAs in sperms, similar to findings observed in *DNAAF2/KTU* mutant individuals [15]. Although it was previously shown that knockout of *Pih1D3* in mice results only in sperm defects, here we show that human *PIH1D3*, located on the X chromosome, encodes a dynein axonemal assembly factor in respiratory cilia and sperm flagella [42]. *PIH1D3* loss-of-function mutations cause classic PCD phenotype with ciliary and sperm immotility in humans.

SUPPLEMENTAL DATA

Supplemental data include five figures, three tables, and six movies and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2016.11.019.

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WEB RESOURCES

Expression Atlas, http://www.ebi.ac.uk/gxa/home
The Human Protein Atlas, http://www.proteinatlas.org/
Online Mendelian Inheritance in Man (OMIM), http://omim.org/
Varbank, https://varbank.ccg.uni-koeln.de/
ExAC Browser, http://exac.broadinstitute.org
Genbank, http://www.ncbi.nlm.nih.gov/genbank

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4

DIAGNOSTIC YIELD OF A TARGETED GENE PANEL IN DUTCH PRIMARY CILIARY DYSKINESIA PATIENTS

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ABSTRACT

We evaluated the expression profile of genes related to primary ciliary dyskinesia (PCD) during *in vitro* ciliogenesis. We selected candidate genes with a similar expression profile and tested the diagnostic yield of targeted-exome sequencing in a Dutch cohort of PCD patients. Twelve healthy non-smoking adults underwent nasal epithelial curette biopsies. We determined differential gene expression in ciliated cells compared to epithelial precursor cells by RNA sequencing. A targeted panel of 26 PCD-related genes and 284 candidate genes with significant upregulation in ciliated cells was sequenced in 74 Dutch PCD patients. All genes reported as PCD causative, except *NME8*, showed significant upregulation during *in vitro* ciliogenesis of human airway cells.

We observed a 67.6% diagnostic yield when testing the targeted-exome panel in a Dutch cohort of 74 patients. The candidate genes included two recently published PCD-related genes *DNAJB13* and *PIH1D3*; identification of the latter was a direct result of this study.We demonstrate a highly sensitive and moderately specific approach for identification of PCD-related genes, based on significant gene upregulation during *in vitro* ciliogenesis. Targeted-exome sequencing had a diagnostic yield of 67.6% in a Dutch PCD population with relatively high percentage of *DNAI and HYDIN* mutations.

INTRODUCTION

Primary ciliary dyskinesia (PCD) is a rare inherited disorder, characterized by dysfunction of motile cilia that line the respiratory tract, fallopian tubes and embryonic node [1]. PCD patients subsequently suffer from recurrent airway infections, situs abnormalities and sub– or infertility [2]. Early diagnosis is important to improve respiratory outcome [3–6]. However, PCD is underdiagnosed or diagnosed late due to unawareness of physicians and complex diagnostic investigations, requiring expensive infrastructure and an experienced team [7, 8]. Diagnosis is primarily based on a combination of evaluation of ciliary ultrastructure by transmission electron microscopy (TEM) and motility by high-speed videomicroscopy (HSVM) [9, 10]. As the number of identified PCD-related genes increases, the North American Genetic Disorders of Mucociliary Clearance Consortium recently proposed a more central role for genetic testing in the diagnostic pathway [11].

Motile cilia have a complex ultrastructure composed of microtubule doublets connecting to multi-protein complexes, such as dynein arms, radial spokes and nexin-dynein regulatory complexes [1]. The axoneme comprises of > 200 distinct proteins [12]. Theoretically, defects in any one of the proteins required to build or regulate the cilium may cause PCD [13]. Therefore, many efforts currently aim to unravel the entire ciliome. Several databases have incorporated results of proteomic, transcriptomic and comparative genomic studies, of which the Cildb is the most comprehensive, covering 55 high-throughput studies in 32 species with cilia or flagella [14]. Regarding these incorporated human ciliary studies, most of the currently known PCD-related genes have been captured by investigating differential gene expression during *in vitro* ciliogenesis of human epithelium using an air-liquid interface (ALI) culture system [15]. This demonstrates that a cell culture system can be a unique model to examine the factors and pathways that regulate mucociliary differentiation, to potentially identify novel PCD genes. As the proportion of ciliated cells that grow in ALI cultures may be less than in human bronchial epithelium, the motile cilia signature may be underestimated with this approach, providing a possible explanation for the lack of detection of several PCD-related genes [16]. In addition to using a candidate gene approach that has led to identification of various PCD-related genes, the widespread use of next generation sequencing (NGS) allowed a large part of recent gene discovery. The extensive genetic heterogeneity in PCD makes NGS approaches also attractive for use in the diagnostic setting, as it offers parallel sequencing of multiple genes or of the entire exome. The diagnostic yield of targeted NGS panels used in Europe and the US currently varies between 43-76% [17–19]. This means that genetic testing cannot yet provide enough certainty to be used as the sole test for PCD. However, considering the pace of new discoveries and advances in sequencing technologies and data analysis, it is reasonable to believe that genetic analysis will be established as the

preferred diagnostic tool in the future. Genetic characterization of international PCD cohorts is pivotal to achieve this. Genetic testing may contribute to the confirmation of PCD diagnosis in patients that have been diagnosed solely based on clinical symptoms or in which results remain inconclusive. Further, genotyping facilitates evaluation and follow-up of genotype-phenotype relationships and adequate genetic counseling of families [20–22]. In this study we therefore aimed to determine the genetic defects in Dutch PCD patients with a targeted gene panel. The gene panel included 26 PCD-related genes known at the beginning of this study and a set of candidate genes. To prioritize the PCD candidate genes we investigated the transcriptome of human airway cells in a monolayer-suspension cell culture, which is aimed at culturing ciliated cells. We hypothesized that PCD-related genes show a distinct expression profile with significant upregulation during ciliogenesis. We tested this proof of principle by using RNA sequencing to analyse global mRNA expression in human airway cells of healthy individuals in a sequential monolayer-suspension cell culture system in which the mRNA expression profile of ciliary cells, grown in suspension, was compared with unciliated precursor cells, grown as monolayer on collagen.

METHODS AND MATERIALS

Subjects

Twelve healthy non-smoking adults were included to undergo nasal curette biopsies for studying differential gene expression during *in vitro* ciliogenesis. All Dutch university hospitals and the PCD patient organisation were requested to inform patients about the study. Participants received detailed information from the researcher upon request. PCD patients from the town of Volendam were excluded as a founder mutation has been identified recently [23]. Informed consent was obtained from all participants prior to study procedures. Studies were approved by the medical ethical committee of the VU University Medical Center.

PCD diagnosis

PCD diagnosis has evolved quickly in the past years, so not all patients have been diagnosed in a similar way [24]. Therefore we classified patients according to European Respiratory Society guidelines as "definite PCD" when they had clinical symptoms and an ultrastructural defect, observed by TEM [10]. Patients with clinical symptoms, low nasal nitric oxide and/or with a motility defect observed by HSVM were classified as "probable PCD" and patients with a previous diagnosis based on only clinical symptoms classified as "PCD clinical diagnosis" [24]. We compared diagnostic status of patients before and after genetic testing.

In vitro ciliogenesis

An experienced pediatric pulmonologist collected nasal epithelial cells from 12 healthy, non-smoking adult subjects [25]. Cells were cultured using a sequential monolayer-suspension culture technique, adapted from Jorissen et al. [26]. For this, cells were seeded on 0.2% rat tail collagen matrix followed by culturing in monolayer medium consisting of Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture media supplemented with 2% Ultroser G, 1% Glutamax and 1% penicillin/streptomycin at 37ºC and 5% CO_2. The monolayer medium promoted the growth of precursor cells without cilia (monolayer phase). After two weeks of culture, cells reached confluency. Subsequently, the collagen matrix was digested with Clostridium histolyticum collagenase to detach the cell layer. The cells were then cultured in suspension medium consisting of Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture media supplemented with 10% NU serum, 1% Glutamax and 1% penicillin/streptomycin on a shaking platform at 55tpm for 1 week. The cells were allowed to grow for 2 more weeks in suspension media during which they gradually became fully ciliated (suspension phase). Ciliogenesis was confirmed by microscopic examination. HSVM was used to examine ciliary function with regard to frequency, amplitude as well as intracellular and intercellular coordination.

Cell harvest and RNA isolation

Cells from 12 subjects were harvested at 2 different time points during the monolayer-suspension cell culture to isolate RNA for RNA sequencing analysis: (T1) on day 14 (unciliated cells in monolayer phase) and (T2) on day 42 (28 days after re-differentiation switch (suspension phase); visible ciliated cells) (figure 1). Normal ciliary beat frequency and beat pattern at T2 were confirmed by HSVM prior to RNA isolation. Cells were centrifuged at 300g for 10min after which the cell pellet was resuspended in 1ml Earle's Balanced Salt Solution. After centrifugation at 300g for 2min the cell pellet was used to isolate RNA with the Quick-RNA Miniprep kit (ZYMO Research, Irvine, CA, USA), according to the manufacturer's protocol. RNA concentration and quality of the RNA samples was determined using the NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA) and/or TapeStation 2200 (Agilent technologies, Santa Clara, CA, USA). RNA was stored at -80°C until further processing.

Gene expression analysis by RNA sequencing

RNA samples were prepared for sequencing using the TruSeq Stranded Total mRNA Library Prep Kit (Illumina Inc. San Diego, USA) according to the manufacturer's instruction. Quality of the cDNA libraries was accessed using TapeStation 2200 (Agilent technologies, Santa Clara, CA, USA). Bar-coded libraries were pooled and run on an Illumina HiSeq 2500 (San Diego, CA, USA) for sequencing. Sequencing reads were cleaned by 5'-end quality trimming and

Illumina-adapter clipping by Trimmomatic [27]. Pre-alignment quality control of the cleaned sequencing reads was done with FastQC [28]. Spliced alignment to reference genome hg19/GRCh37 of cleaned sequencing reads was done with STAR, guided by gene annotation in the refGene UCSC table [29]. Post-alignment quality control including genebody coverage analysis was done with RSeQC [30]. Read summarization was performed with HTseq using strandness-aware union intersection of uniquely aligned reads (mapping quality threshold 35) with refGene gene annotation [31]. A pre-normalization filter was used excluding genes that had less than 5 reads in more than 80% of the samples. Using edgeR, trimmed mean of M-values (TMM) normalization was performed followed by differential expression between monolayer (cilium deficient) and suspension (cilium proficient), paired by patient using a generalized linear model [32]. Genes with false discovery rate (FDR) adjusted p-values <0.05 and logCPM >0 were considered significant. Downstream analyses and visualizations of differential gene expression including heat maps and boxplots were performed using R version 3.2.2.

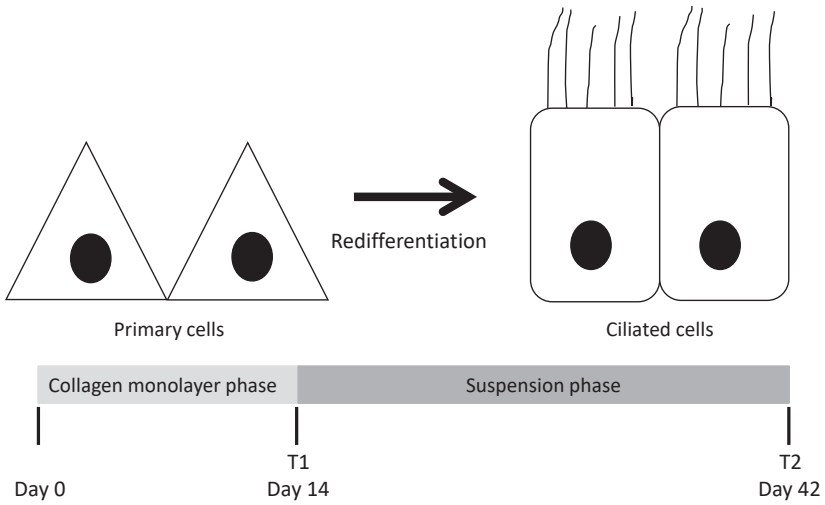

Figure 1. Schematic overview of in vitro ciliogenesis

Schematic overview of in in vitro ciliogenesis using a collagen monolayer-suspension cell culture system. RNA was isolated at T1 (after 14 days) and at T2 (after 42 days).

DNA extraction

Saliva from PCD patients was collected using Oragene-250 self-collection saliva kits (DNA Genotek Inc., Canada) that were delivered to patients' homes. DNA extraction was performed according to the manufacturer's protocol. Briefly, the collected cells in the saliva were subjected to lysis using a purifying buffer provided in the kit to remove proteins. This was followed by by incubation on ice and DNA precipitation with 100% ethanol. The DNA

was rehydrated in TE (Tris–HCl 1 mM pH 8.0 and 0.5 mM EDTA pH 8.0). DNA quantity and quality were measured by ultraviolet spectrometry with the NanoDrop 1000 (Thermo Fisher Scientific, USA). DNA samples were stored at -20°C.

Targeted gene panel

Twenty-six PCD-related genes that were known at the time of composing the gene panel were included (table S4.1). Additional candidate genes were selected from the DEG list based on showing a clear link to ciliary or flagellar proteins or processes in published candidate gene lists and the Gene Network database (table S4.1) [14].

DNA Sequencing

DNA was sheared by sonification to an average size of 200 bp with the Covaris S220 (Covaris, Woburn, MA, USA). Sheared DNA was subjected to Illumina paired-end DNA library preparation and enriched for target sequences (MYbaits Target Enrichment kit, MYcroarray, USA). Target sequences included the 310 genes listed in table S4.1 or the entire exome combined with in silico analysis of the genes listed. Enriched libraries were sequenced with the HiSeq 2500 platform (Illumina, USA) as paired-end 100 bp reads. Sequencing reads were cleaned by 5'-end quality trimming and Illumina-adapter clipping by Trimmomatic [27]. Pre-alignment quality control of the cleaned sequencing reads was done with FastQC [28]. Clean reads were mapped to reference genome hg19 (GRCh37) using BWA-MEM. The genome analysis toolkit was used for recalibrating quality scores, realignment around indels, marking PCR duplicates and variant calling and were annotated with ANNOVAR.

Analysis of variant pathogenicity after sequencing

Filtering of variants for novelty was performed by exclusion of variants with frequency >0.01 in the 1000 Genomes database or the Exome Sequencing Project (ESP). We focused on nonsynonymous mutations, frame-shift mutations, splice-site mutations and indels following an autosomal-recessive or X-linked inheritance pattern. Variants in one of the PCD-related genes were considered disease-causing when they correlated with ultrastructural and/or ciliary motility defect observed by electron microscopy and HSVM, respectively. Expected pathogenicity of nonsynonymous mutations was evaluated by a combination of prediction scores such as PolyPhen, SIFT and Mutation Taster. The predicted effect of splice-site mutations (Alamut Visual, Interactive Biosoftware, USA) were confirmed in cDNA from respiratory epithelial cells after sequential monolayer-suspension culture whenever possible. For this RNA was isolated as previously described. cDNA syhthesis was performed with the SuperScript® VILO cDNA Synthesis Kit (Thermo fisher Scientifi Inc., Waltham, USA) according to manufacturer's instructions.

Analysis of variants in *HYDIN*

As *HYDIN* has a pseudogene on chromosome 1 (*HYDIN2*) with very high homology to exon 6-83, we confirmed likely pathogenic *HYDIN* variants, occuring in around 25% or 50% of sequence data, with allele-specific PCRs. We designed the primers by aligning *HYDIN* and *HYDIN2* genomic DNA sequences in BLAST to have a single base pair difference at the 3' ends [33]. Primer sequences are presented in table S4.2. We expected that sequencing reads that could be mapped to both the *HYDIN gene* and the *HYDIN2* gene would automatically be discarded. We re-mapped the sequencing data to the reference genome after removing the *HYDIN2* gene from this genome on chromosome 1 to investigate whether this method allows identification of additional variants that would have been missed by standard analysis. Suspected variants were resequenced with Sanger sequencing, using PCR primers specific for the active gene on chromosome 16.

RESULTS

Differential gene expression during in vitro ciliogenesis using RNA sequencing

RNA samples from one healthy control did not pass quality control steps and were discarded from the analysis. Two differentially expressed gene clusters ($p<0.05$, FDR<0.05) were identified (figure 2). One cluster (5198/25963, 20.0 %) included genes with increased expression during the monolayer phase of cell culture (T1) and the other cluster included genes with increased expression during the suspension phase (T2) (5499/25963, 21.2%).The latter cluster included 35 of 36 (all but *NME8*) currently known PCD-related genes (figure 3, table S4.3). In contrast to other PCD-related genes, *NME8* showed no expression using RNA sequencing of human airway cells in 12 healthy controls. All other PCD-related genes either showed relatively low expression before ciliogenesis and high expression during ciliogenesis or a relatively high expression throughout the entire experiment, whereas *NME8* showed no expression throughout the entire experiment. We propose this gene cluster can be best used in combination with exome data to prioritize analysis of gene variants that may play an important role in ciliogenesis, thereby aiding PCD gene discovery.

Diagnostic yield of the targeted exome panel

A total of 83 PCD patients applied to participate in this study, of which 74 were included (figure 4). In 50/74 patients (67.6%) we found biallelic mutations that are expected to be pathogenic in a total of 12 PCD-related genes, including in the recently identified *PIH1D3* gene (table 1, figure 5). Most gene defects were found in *DNAH5* (13/74 patients, 17.6%) and *DNAI1* (12/74 patients, 16.2%). The relatively high proportion of *DNAI1* mutations in our cohort appears largely due to the common c.48+2dup (also known as IVS1+2insT) founder

mutation that was present in 15/24 (62.5%) alleles in patients with biallelic mutations in *DNAI1*. Further, we observed a very high frequency (6/74, 8.1%) of *HYDIN* mutations in our population. We used *HYDIN*-specific primers to confirm likely pathogenic mutations. Re-mapping sequencing data, after removing the pseudogene (*HYDIN2)* from the reference genome resulted in two additional variants that would otherwise have been missed. Further, we identified a hemizygous mutation in the novel X-linked gene *PIH1D3* in a male with PCD without syndromic coseggregation, which has recently been published [34]. Among the 284 selected candidate genes in our gene panel was also *DNAJB13*, which has only recently been identified as a novel PCD-related gene [35]. In 7/74 patients (9.5%) we only observed monoallelic mutations in currently known PCD-related genes (table 2). In these cases we may have missed deep intronic mutations that may lead to splice defects. In 17/74 patients (23.0%) the analysis revealed no disease-causing mutations. These patients all had multiple nonsynonymous variants of unknown significance (VUS) in multiple PCD-related genes and/or candidate genes. Figure 4 gives an overview of the diagnostic classification of PCD patients before and after genetic testing.

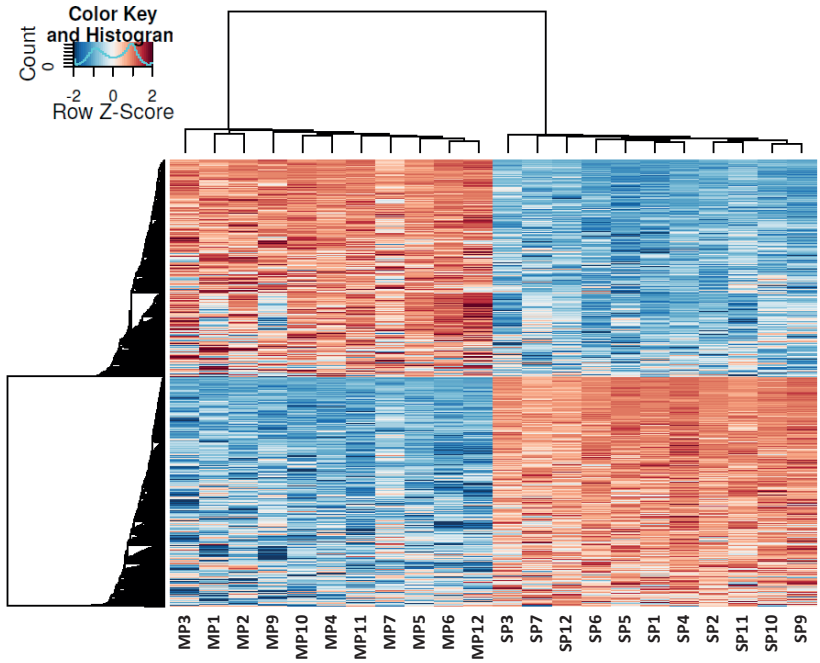

Figure 2. Heatmap of gene expression during in vitro ciliogenesis

Heat map showing gene expression in human respiratory epithelial cells during collagen monolayer-suspension cell culture. Changes at two different timepoints (T1: monolayer phase and T2: suspension phase) of 11 samples are depicted. Red colour indicates high expression and blue indicates low expression. MP: monolayer phase; SP: suspension phase.

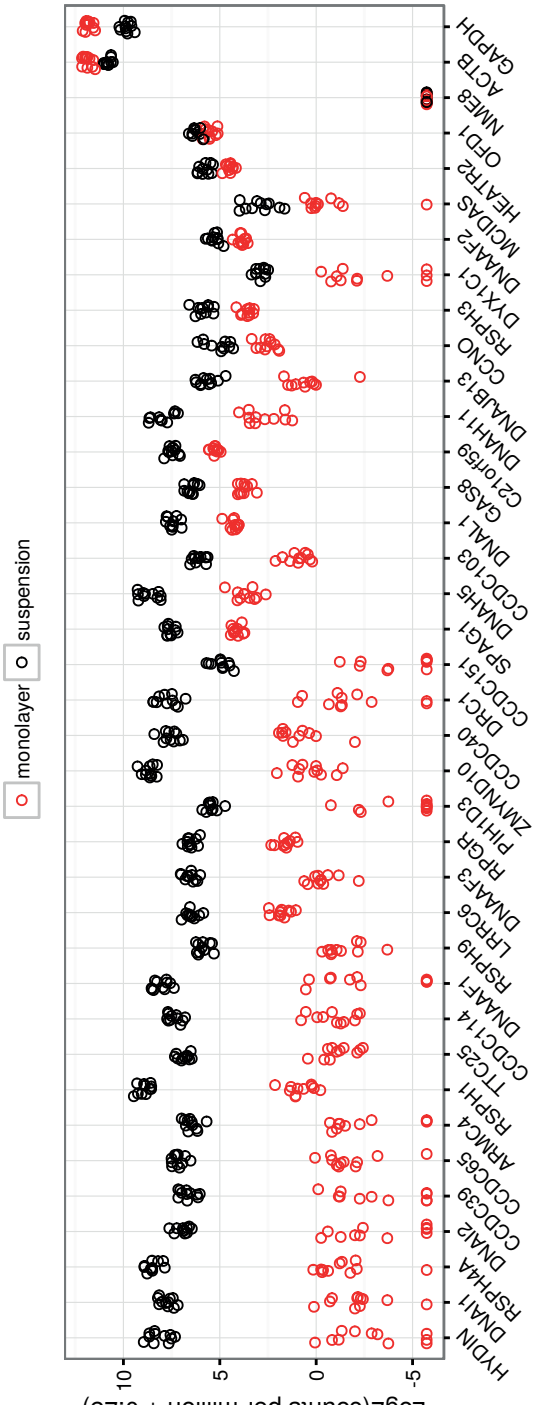

Figure 3. Grid of gene expression of PCD-related genes during in vitro ciliogenesis

Graphical representation of gene expression for all PCD-related genes. Levels of gene expression in 11 samples of human respiratory epithelial cells are shown in red at T1 (monolayer phase) and in black at T2 (suspension phase) on a log2 (counts per million) scale. ACTB and GAPDH are added to compare gene expression of household genes.

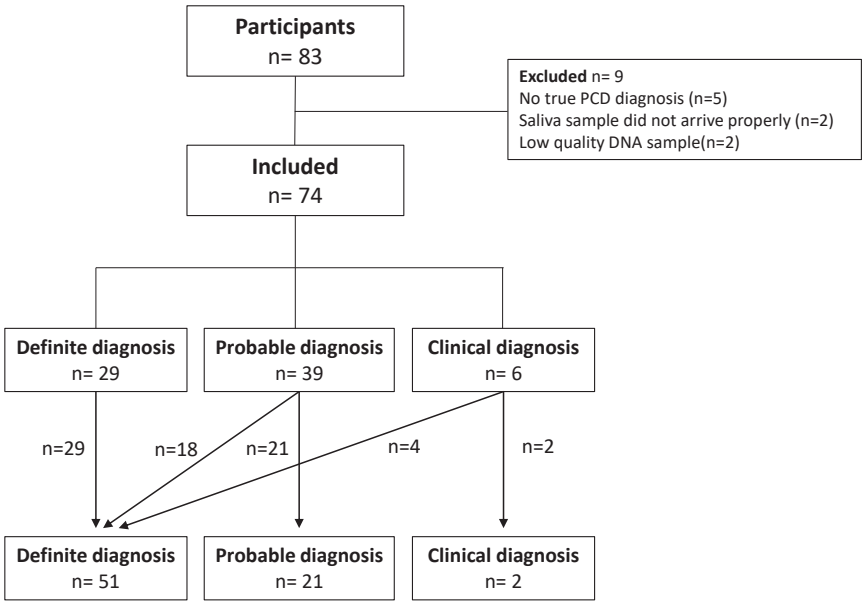

Figure 4. Overview of diagnostic certainty in PCD patients before and after genetic testing.

Overview of diagnostic certainty in PCD patients included in the study, before and after genetic testing. Definite diagnosis: hallmark ultrastructural defect and/or biallelic pathogenic mutation; Probable diagnosis: hallmark ciliary motion defect and/or low nasal nitric oxide; Clinical diagnosis: only clinical PCD symptoms.

Table 1. Overview of biallelic mutations related to PCD diagnosis.

Patient ID	Gene	Variants and protein change	Effect	Zygosity	Motion defect	Ultrastructural defect	Ref.
PCD-2035	DNAH5	c.10384C>T:p.Q3462X; c.10815delT:p.D3606Hfs*23	Stopgain; Frameshift deletion	HET	Unknown	ODA + IDA	[17, 36]
PCD-0106	DNAH5	c.4664G>A:pW1555X; c.376delG:p.V126Yfs*23	Stopgain; Frameshift deletion	HET	Immotile	ODA + IDA	Novel
PCD-0032	DNAH5	c.10384C>T:p.Q3462X; c.13338+5G>A	Stopgain; Splicing	HET	Immotile	ODA + IDA	[17, 36]
PCD-0031	DNAH5	c.13729C>T:p.R4577X; c.3989_3990del:p.E1330Afs*3	Stopgain; Frameshift deletion	HET	Immotile	ODA + IDA	Novel
PCD-0103	DNAH5	c.5495T>A:p.L1832X; c.10815delT:p.D3606Hfs*23	Stopgain; Frameshift deletion	HET	Immotile	ODA	Novel; [36]
PCD-0118	DNAH5	c.10384C>T:p.Q3462X; c.8368delT:p.Y2790Mfs*16	Stopgain; Frameshift deletion	HET	Immotile	ODA + IDA	[17]; Novel
PCD-2040	DNAH5	c.2578-1G>A; c.10815delT:p.D3606Hfs*23	Splicing; Frameshift deletion	HET	Immotile	ODA	Novel; [36]
PCD-2057	DNAH5	c.10384C>T:p.Q3462X; c.4360C>T:p.R1454X	Stopgain; Stopgain	HET	Unknown	ODA + IDA	[17, 37]
PCD-3058 [Sib. 3059]	DNAH5	c.1089+1G>A; c.5177T>C:p.L1726P	Splicing; Nonsynonymous	HET	Immotile	ODA+IDA	[17, 38]
PCD-3059	DNAH5	c.1089+1G>A; c.5177T>C:p.L1726P	Splicing; Nonsynonymous	HET	Immotile	ODA + IDA	[17, 38]
PCD-1162	DNAH5	c.5647C>T:p.R1883*; c.1852C>T:p.R618*	Stopgain; Stopgain	HET	Immotile	ODA	[38]; Novel
PCD-3152 [Sib. 3153]	DNAH5	c.1730+2T>C; c.1089+1G>A	Splicing; Splicing	HET	Immotile	Unknown	Novel; [38]
PCD-3153	DNAH5	c.1730+2T>C; c.1089+1G>A	Splicing; Splicing	HET	Immotile	ODA	Novel; [38]
PCD-0051	DNAI1	c.48+2dup; c.48+2dup	Splicing; Splicing	HOM	Immotile	ODA + IDA	[39]

Table 1. Overview of biallelic mutations related to PCD diagnosis. *(Continued)*

Patient ID	Gene	Variants and protein change	Effect	Zygosity	Motion defect	Ultrastructural defect	Ref.
PCD-0108	DNAI1	c.48+2dup; c.48+2dup	Splicing; Splicing	HOM	Low frequency, stiff	ODA	[39]
PCD-0154	DNAI1	c.48+2dup; c.48+2dup	Splicing; Splicing	HOM	Immotile	ODA	[39]
PCD-3146	DNAI1	c.48+2dup; c.48+2dup	Splicing; Splicing	HOM	Low frequency, stiff	ODA	[39]
PCD-8073	DNAI1	c.48+2dup; c.48+2dup	Splicing; Splicing	HOM	Unknown	Unknown	[39]
PCD-0102	DNAI1	c.48+2dup; c.1229delT:p.I410Afs*61	Splicing; Frameshift deletion	HET	Immotile	ODA	[39]; Novel
PCD-5164	DNAI1	c.48+2dup; c.1031+5G>T	Splicing; Splicing	HET	Immotile	Unknown	[39]; Novel
PCD-5165 [Sib.5164]	DNAI1	c.48+2dup; c.1031+5G>T	Splicing; Splicing	HET	Low frequency, stiff	ODA	[39]; Novel
PCD-1136	DNAI1	c.814C>T:p.Q272X; c.814C>T:p.Q272X	Stopgain; Stopgain	HOM	Unknown	Unknown	Novel
PCD-1137 [Sib. 1136]	DNAI1	c.814C>T:p.Q272X; c.814C>T:p.Q272X	Stopgain; Stopgain	HOM	Almost no cilia, immotile	Unknown	Novel
PCD-8170	DNAI1	c.48+2dup; c.577_578delAC:p.T193*	Splicing; Frameshift deletion	HET	Immotile	ODA + IDA	[39]; Novel
PCD-8076	DNAI1	c.48+2dup; c.1243delT:p.Y415Tfs*56	Splicing; Frameshift deletion	HET	Immotile	Unknown	[39]; Novel
PCD-0029	HYDIN	c.6669+1G>A; c.6669+1G>A	Splicing; Splicing	HOM	Low frequency, stiff, lack of coordination	Normal	Novel
PCD-0043	HYDIN	c.8356C>T:p.R2786*; c.13867G>T:p.G4623*	Stopgain; Stopgain	HET	Low frequency, stiff, circular movement	Normal, some single tubuli	Novel
PCD-1124	HYDIN	c.5388C>A:p.Y1796*; c.12444-3C>G	Stopgain; Splicing	HET	Low frequency, stiff, lack of coordination	Normal, some single tubuli	Novel

Table 1. Overview of biallelic mutations related to PCD diagnosis. *(Continued)*

Patient ID	Gene	Variants and protein change	Effect	Zygosity	Motion defect	Ultrastructural defect	Ref.
PCD-8179	HYDIN	c.8674_8675delinsG:p.Q2892Gfs*3; c.8674_8675delinsG:p.Q2892Gfs*3	Frameshift delins; Frameshift delins	HOM	Low frequency, stiff, lack of coordination	Normal	Novel
PCD-8078 [Sib.8179]	HYDIN	c.8674_8675delinsG:p.Q2892Gfs*3; c.8674_8675delinsG: p.Q2892Gfs*3	Frameshift delins; Frameshift delins	HOM	Low frequency, stiff, lack of coordination	Unknown	Novel
PCD-8184	HYDIN	c.8674_8675delinsG: p.Q2892Gfs*3; c.8674_8675delinsG: p.Q2892Gfs*3	Frameshift delins; Frameshift delins	HOM	Low frequency, stiff, lack of coordination	Normal	Novel
PCD-2060	DNAAF1	c.811C>T:p.R271*; c.811C>T:p.R271*	Stopgain; Stopgain	HOM	Immotile	ODA + IDA	[40]
PCD-3044	DNAAF1	c.1528+2T>C; c.1528+2T>C	Splicing; Splicing	HOM	Low frequency, stiff, lack of coordination	ODA + IDA	Novel
PCD-3045 [Sib. 3044]	DNAAF1	c.1528+2T>C; c.1528+2T>C	Splicing; Splicing	HOM	Low frequency, stiff, lack of coordination	ODA + IDA	Novel
PCD-4067	DNAAF1	c.1528+2T>C; c.1528+2T>C	Splicing; Splicing	HOM	Low frequency, stiff, lack of coordination	ODA + IDA	Novel
PCD-0142	DNAH11	c.10174C>T:p.R3392C; c.12889C>T:p.R4297W	Nonsynonymous; Nonsynonymous	HET	High frequency, stiff	Normal	Novel; Novel
PCD-0038	DNAH11	c.9824A>C:p.Y3275S; c.13304-1G>A	Nonsynonymous; Splicing	HET	High frequency, stiff	Normal	Novel; Novel
PCD-8181	DNAH11	c.793_794insCAGCT:p.R265Pfs*5; c.10568+1G>A	Frameshift insertion; Splicing	HET	High frequency, stiff	Normal	Novel; Novel
PCD-2061	DNAH11	c.7913A>G: p.Q2638R; c.7913A>G: p.Q2638R	Nonsynonymous; Nonsynonymous	HOM	High frequency, stiff	Normal	Novel
PCD-0101	CCDC103	c.461A>C:p.H154P; c.461A>C:p.H154P	Nonsynonymous; Nonsynonymous	HOM	Low frequency, stiff, lack of coordination	ODA	[41]
PCD-0115	CCDC103	c.461A>C:p.H154P; c.461A>C:p.H154P	Nonsynonymous; Nonsynonymous	HOM	Low frequency, stiff, lack of coordination	ODA + IDA	[41]
PCD-0009	CCDC40	c.1677_1678insAC:p.E559Rfs*11; c.1677_1678insAC:p.E559Rfs*11	Frameshift insertion; Frameshift insertion	HOM	Low frequency, stiff, lack of coordination	IDA + oriëntation	Novel

Table 1. Overview of biallelic mutations related to PCD diagnosis. *(Continued)*

Patient ID	Gene	Variants and protein change	Effect	Zygosity	Motion defect	Ultrastructural defect	Ref.
PCD-0110	CCDC40	c.248delC:p.A83Vfs*84; c.C1855T:p.Q619*	Frameshift deletion; Stopgain	HET	Low frequency, stiff, lack of coordination	Oriëntation	[42]; Novel
PCD-0128	CCDC39	c.2347_2351del:p.F783Yfs*3; c.2347_2351del:p.F783Yfs*3	Frameshift deletion; Frameshift deletion	HOM	Low frequency, stiff,	IDA	Novel
PCD-3163	CCDC39	c.610-2A>G; c. -13 to c.7del	Splicing; Splicing	HET	Low frequency, stiff, lack of coordination	Oriëntation	[43]; Novel
PCD-0050	DNAAF2	c.1901T>C:p.F634S; c.998 C>T:p.A333V	Nonsynonymous; Splicing	HET	Immotile	ODA + partial IDA	Novel; Novel
PCD-3147	HEATR2	c.50_51insG:p.E19Gfs*5; c.1499G>T:p.C500F	Frameshift insertion; Nonsynonymous	HET	Low frequency, lack of coordination	ODA + IDA	Novel; Novel
PCD-3148 [Sib. 3147]	HEATR2	c.50_51insG:p.E19Gfs*5; c.1499G>T:p.C500F	Frameshift insertion; Nonsynonymous	HET	Low frequency, lack of coordination	Unknown	Novel; Novel
PCD-0030	CCNO	c.787insG:pR263Afs; c.787insG:pR263Afs	Frameshift insertion; Frameshift insertion	HOM	No cilia	No cilia	Novel
PCD-8083	PIH1D3	c.357_363delGGTGGGA:p.V120Lfs*6	Frameshift deletion	HEMI	Immotile	ODA + IDA	[34]

Overview of biallelic mutations and observed defects in ciliary motion and ultrastructure in PCD patients. HOM: homozygous; HET: heterozygous; HEMI: hemizygous; ODA: outer dynein arm; IDA: inner dynein arm; Ref.: reference. DNAH5 (NM_001369.2), DNAI1 (NM_001281428.1), HYDIN (NM_001270974.2), DNAAF1 (NM_178452.5), DNAH11 (NM_001277115.1), CCDC103 (NM_213607.2), CCDC40 (NM_017950.3), CCDC39 (NM_181426.1), HEATR2 (NM_017802.3), DNAAF2 (NM_018139.2), CCNO (NM_021147.3), PIH1D3 (NM_001169154.1).

Table 2. Overview of monoallelic mutations potentially related to PCD diagnosis

Patient ID	Gene	Variant and protein change	Effect	Zygosity	HSVM defect	EM defect	Ref.
PCD-8072	DNAI1	c.48+2dup; [unknown]	Splicing	HET	Immotile	CP defect, partial IDA	[39]
PCD-8077	DNAI1	c.48+2dup; [unknown]	Splicing	HET	Unknown	Unknown	[39]
PCD-1123	DNAH11	c.7279C>G:p.Q2426E; [unknown]	Nonsynonymous	HET	Lack of coordination	Normal	Novel
PCD-1127	DNAH11	c.3291C>A:p.S1097R; [unknown]	Nonsynonymous	HET	Lack of coordination	Normal	Novel
PCD-0041	CCDC39	c.1960G>A:pG654R; [unknown]	Nonsynonymous	HET	Low frequency, stiff, lack of coordination	Partial IDA	Novel
PCD-7171	HYDIN	c.14625_14626delGC:pP4876Lfs*16; [unknown]	Frameshift deletion	HET	Low frequency, stiff, lack of coordination	Normal	Novel
PCD-0011	HYDIN	c.14625_14626delGC:pP4876Lfs*16; [unknown]	Frameshift deletion	HET	Stiff	Normal	Novel

Overview of monoallelic mutations and observed defects in ciliary motion and ultrastructure in PCD patients. HOM: homozygous; HET: heterozygous; HEMI: hemizygous; ODA: outer dynein arm; IDA: inner dynein arm; Ref.: reference. DNAI1 (NM_001281428.1), DNAH11 (NM_001277115.1), CCDC39 (NM_181426.1), HYDIN (NM_001270974.2).

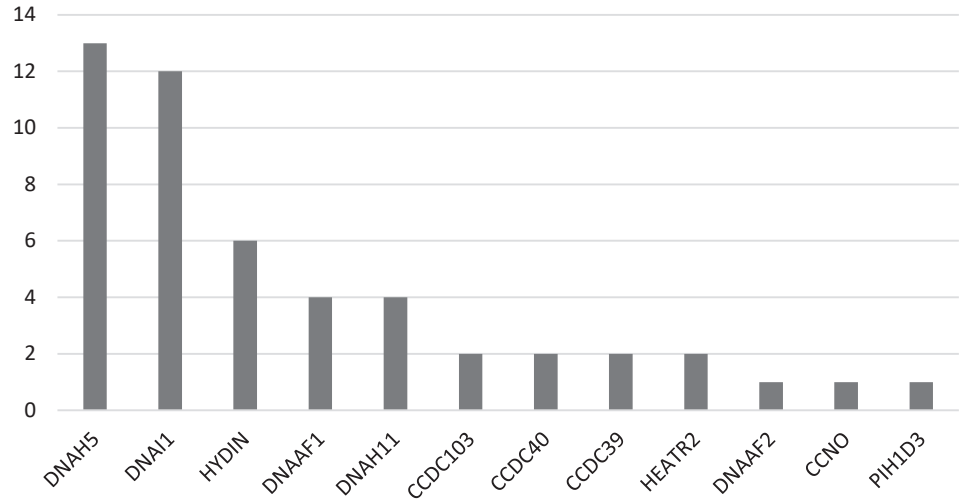

Figure 5. Overview of PCD-related genes in which bi-allelic pathogenic mutations were found in 74 Dutch PCD patients.

Data are depicted as numbers. DNAH5 (NM_001369.2), DNAI1 (NM_001281428.1), HYDIN (NM_001270974.2), DNAAF1 (NM_178452.5), DNAH11 (NM_001277115.1), CCDC103 (NM_213607.2), CCDC40 (NM_017950.3), CCDC39 (NM_181426.1), HEATR2 (NM_017802.3), DNAAF2 (NM_018139.2), CCNO (NM_021147.3), PIH1D3 (NM_001169154.1).

DISCUSSION

In the present study, we observed that all but one PCD-related gene (*NME8*) showed significant upregulation during *in vitro* ciliogenesis of human respiratory cells. We demonstrate that the identified differentially expressed gene cluster including ~ 5500 genes is highly sensitive for detection of currently known PCD-related genes, but also involves many unrelated genes. When using a targeted exome panel including a set of 26 PCD-related genes and 284 selected candidate genes, we observed a diagnostic yield of 67.6% in 74 Dutch PCD patients. The candidate genes included two recently identified PCD-related genes: *DNAJB13* and *PIH1D3*, of which the latter was a direct discovery of the current study [34, 35]. This underlines the value of using differentially expressed genes during *in vitro* ciliogenesis of human airway cells, to prioritize PCD candidate genes.

Differentially expressed gene cluster

In comparison to other human ciliary studies included in the Cildb, the DEG cluster analysis of ciliogenesis gene expression presented here is the only approach that detected nearly all currently known PCD-related genes. The unique aspect of our approach is the comparison of gene expression during in vitro ciliogenesis with gene expression in the precursor cells,

allowing us to identify upregulated genes. A cluster of ~5500 genes cannot be easily incorporated in a targeted-exome sequencing gene panel due to its large size. However, this cluster analysis as an *in silico* gene panel after whole exome sequencing, is a potentially powerful tool to prioritize variants in novel candidate PCD-related genes in patients that do not harbour pathogenic variants in any of the currently known PCD-related genes. This would also reduce the number of variants to investigate to approximately 21% of complete whole-exome data.

NME8

Interestingly, *NME8* gene expression during *in vitro* ciliogenesis was different from other PCD-related genes, with a lack of expression throughout the entire culture. Other PCD-related genes showed either 1) a relatively high expression throughout the two phases of the cell culture with a modest increase in the suspension phase in which ciliogenesis takes place or 2) a relatively low expression in the unciliated monolayer phase of cell culture followed by a major increase in expression during the suspension phase. Duriez and co-workers identified *NME8* (also known as *TXNDC3*) as a candidate PCD gene in 2007 as the human ortholog of the sea urchin gene encodes a component of sperm outer dynein arms [44]. They have described two compound heterozygous mutations in a girl with PCD from consanguineous parents. These include a nonsynonymous variant predicted to be pathogenic and a common variant predicted to lower expression of the *TXNDC3d7* isoform, which is suggested to be detrimental. The distinct behaviour of *NME8* in comparison to other known PCD-related genes could indicate that our approach is not 100% sensitive in identifying genes that are vital for proper ciliary function. The *in vitro* monolayer-suspension cell culture system may not reflect all processes that occur during *in vivo* ciliogenesis. On the other hand one might argue that the mutations described in *NME8* may not be causative of PCD in the described patient. It is unknown whether homozygous mutations, which are highly likely in a consanguiseous family, in other genes with ODA defects have been ruled out in this patient. To our knowledge, this is the only patient reported with possible disease-causing mutations in *NME8*. It is, however, not uncommon given that many private mutations have been reported in PCD.

Diagnostic yield of gene panel

The obtained diagnostic yield of 67.6% in a Dutch PCD cohort is comparable to the yield observed in three other studies in which genetic panels were used in combination with Sanger pre-sequencing or targeted copy number variation (CNV) analysis, aimed at published exon deletions in known PCD-related genes [17–19]. The latter increased the diagnostic yield by 8.9% to 76% in a study by Marshall and co-workers [18]. The current study did not include PCD patients from the town of Volendam. If we take into account that

this town currently has 56 identified PCD patients all harbouring the same homozygous founder mutation, the diagnostic yield is potentially much higher. Our results confirm that the majority of unrelated PCD patients have defects in a core of 5-10 PCD genes, whereas most other mutations are private. Despite this similarity, the distribution of genetic defects is quite different from other countries. *DNAI1*, *CCDC114* and *HYDIN* mutations are much more prevalent in Dutch PCD patients than in others. In case of *DNAI1* and *CCDC114* this is due to founder mutations. To our knowledge, this is the first time that such a high frequency of *HYDIN* mutations is demonstrated. It is unknown whether this is specific to the Dutch population or whether this is a result of the improved extensive analysis of *HYDIN* variants and re-mapping of sequencing data, which prevents automatic discarding of reads which also map to the pseudogene. This proof of concept study remains to be validated in other PCD patient cohorts but definitely offers a novel method to deal with pseudogenes in NGS data analysis.

Variation in ultrastructure and motility defects

There was a slight difference in TEM defects observed in some of the patients with biallelic mutations in the same genes, such as in *DNAH5*. This has been observed in other studies as well and is expected to reflect the difficult evaluation of the IDAs [17]. We also see slight variation in motility defects as a consequence of *DNAAF1* mutations, from complete immotility to severly reduced ciliary beat frequency and amplitude. This has been previously observed in other dynein assembly factors *DNAAF2* and *4*, but it is uncommon in *DNAAF1*.

Unsolved cases

90% of patients in which we did not find biallelic mutations or monoallelic mutations in PCD-related genes had a "likely PCD diagnosis", 5% had "clinical PCD diagnosis" and 5% had a "definite PCD diagnosis". Gene defects in these patients are expected to be identified in one of the 7 currently known PCD-related genes that were not included in the gene panel or in yet to discover PCD-related genes. Another possibility is that some of these individuals may not be true PCD patients. However, this is unlikely as 95% of them repeatedly showed abnormal ciliary beat pattern or ciliary immotility. Finally, many patients had compound heterozygous non-synonymous variants in PCD-related genes, with very low or unknown population frequencies, which may be causative of PCD. The possible causative effect of these variants remains to be studied. It could be reasoned that non-synonymous variants may have relatively small effects in large genes, such as *DNAH5*. On the contrary, however, in other diseases where large genes are affected, such as *FBN1* in Marfan syndrome, the majority of causative mutations is non-synonymous [45].

Genetic testing approach

We cannot yet rely on genetic testing as a sole diagnostic test, as approximately 30% of patient cases remain unsolved [17–19]. Most likely there are only private mutations left to identify in PCD. Therefore, two possible strategies can be proposed to aid genetic testing in diagnostics; 1) a two-tiered approach with a (country specific) targeted gene panel with subsequent exome sequencing in patients in which no mutations are found, or 2) directly applying exome sequencing in all patients. The latter approach is becoming more frequently used as costs lower and coverage homogeneity is improved. When choosing exome sequencing we propose to use *in silico* panels of 1) the 36 currently known PCD-related genes and/or 2) the cluster differentially expressed genes during *in vitro* ciliogenesis of human airway cells, to prioritize variants in possibly novel PCD-related genes. This approach enables pre-selection of variants in genes with a similar expression to currently known PCD-related genes and possible reduction of the total exome data analysis to ~21%.

To summarize, we present a differentially expressed gene cluster with significant upregulation during *in vitro* ciliogenesis of human airway cells. This cluster identified all but one currently known PCD-related genes. From these, only *NME8* exhibited very low expression throughout the entire experiment. Using this gene cluster in the analysis of exome sequencing data may aid in the PCD gene discovery by prioritizing genes that could play an important role in ciliogenesis. Further, we observed a 67.6% diagnostic yield when testing a gene panel of 26 known PCD-related genes and 284 selected candidate genes in a Dutch cohort, not including PCD patients from the Volendam population harbouring a *CCDC114* founder mutation. Dutch PCD patients appear to have a relatively high percentage of *DNAI, CCDC114* and *HYDIN* mutations compared to data from other countries. The candidate genes that were selected from the differentially expressed gene cluster also included two novel PCD-related genes *DNAJB13* and *PIH1D3*, of which identification of the latter was a direct result of this study. This illustrates the potential of the differentially expressed gene cluster approach in the discovery of novel PCD-related genes.

SUPPLEMENTAL DATA

Supplemental data include three tables and can be found with this article online.

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5

BREATHOMICS IN LUNG DISEASE

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ABSTRACT

Volatile organic compounds (VOCs) are produced by virtually all metabolic processes of the body. As such, they have potential to serve as noninvasive metabolic biomarkers. Since exhaled VOCs are either derived from the respiratory tract itself or have passed the lungs from the circulation, they are candidate biomarkers in the diagnosis and monitoring of pulmonary diseases in particular. Good examples of the possibilities of exhaled volatiles in pulmonary medicine are provided by the potential use of VOCs to discriminate between patients with lung cancer and healthy control subjects and to noninvasively diagnose infectious diseases and the association between VOCs and markers of disease activity that has been established in obstructive lung diseases. Several steps are, however, required prior to implementation of breath-based diagnostics in daily clinical practice. First, VOCs should be studied in the intention-to-diagnose population, because biomarkers are likely to be affected by multiple (comorbid) conditions. Second, breath collection and analysis procedures need to be standardized to allow pooling of data. Finally, apart from probabilistic analysis for diagnostic purposes, detailed examination of the nature of volatile biomarkers not only will improve our understanding of the pathophysiologic origins of these markers and the nature of potential confounders but also can enable the development of sensors that exhibit maximum sensitivity and specificity toward specific applications. By adhering to such an approach, exhaled biomarkers can be validated in the diagnosis, monitoring, and treatment of patients in pulmonary medicine and contribute to the development of personalized medicine.

DIAGNOSIS BY OLFACTION

Aristotle taught doctors to use their sense of smell. Liver disease was identified by a fecal breath and stale beer smell suggested the presence of TB [1]. A more recent anecdote comes from Oscar the cat who was able to smell impeding death at a Rhode Island nursing home [2]. Such observations stimulated research on the use of Volatile Organic Compounds (VOCs), the main molecular substrate underlying mammalian olfaction, as biomarkers. This review focuses on the validation of VOCs as novel biomarkers for pulmonary medicine. The rationale behind VOCs, validation of current results and their potential future use and the obstacles that need to be overcome are addressed.

VOCs reflect metabolism in humans

VOCs are gaseous organic molecules that are emitted from the fluid phase because they are highly volatile. Human VOCs are released from skin, with feces, urine and breath and are derived from many metabolic pathways. Since cellular metabolism is altered by disease, the resulting change in VOCs may serve as biomarkers for particular pathophysiological conditions. In pulmonary medicine breath is of special interest because of its intensive contact with the respiratory tract. The rate at which VOCs are exhaled is the net effect of several interacting (bio)chemical processes; intracellular and extracellular degradation, solubility of the compound in extracellular fluid, fat, and blood, the affinity with extracellular matrix and carrier proteins, the concentration gradient with the alveolar and bronchial air, the vapor pressure and alveolar ventilation [3]. This results in a chemical equilibrium of a given compound between breath, blood, and fat that can be described by that substance's physiochemical partition constant [4].

Nobel laureate Pauling and colleagues were the first to isolate > 200 organic volatiles from a single breath sample [5]. To date, several thousands of individual VOCs have been identified, generally occurring in the parts per million/parts per billion range. This multitude of markers likely represents the complexity of human biology more accurately than do isolated biomarkers. "Breathomics" may, therefore, have potential for noninvasive diagnosis and monitoring of disease, which closely fits a personalized medicine approach [6]. As the interest in this field is growing rapidly, this may expedite the development of VOCs in lung disease.

Exhaled VOCs may be of local, systemic or exogenous origins (figure 1). Locally produced compounds diffuse directly into alveoli or the airway lumen along the respiratory tract. Volatiles of systemic origins are derived from the circulation after originating from metabolic processes elsewhere and dissolving into the blood. Therefore, even nonpulmonary diseases contribute to exhaled VOCs, which has successfully been used in the assessment of

nonpulmonary malignancies [7]. Exogenous VOCs can be inhaled or absorbed through the skin. They primarily originate from nonhuman sources and exist in three categories. First, VOCs introduce undesired noise by being inspired and expired without interaction with the body. A second group of exogenous VOCs does interact with human tissue and can be stored inside the body for extensive periods of time [3]. The latter volatiles can, therefore, serve as potential biomarkers for environmental exposures and buildup of toxins, such as cigarette smoke carcinogen *N*-Nitrosomine [8]. The third group of exogenous VOCs is of (resident) microbial origin (predominantly bacteria, but also fungi and viruses), making them of specific interest when identifying infectious diseases or diseases linked to changes in microbiome [9]. Since VOCs reflect this broad range of pathophysiological processes they have the potential to meet many of the criteria for an ideal biomarker (table 1).

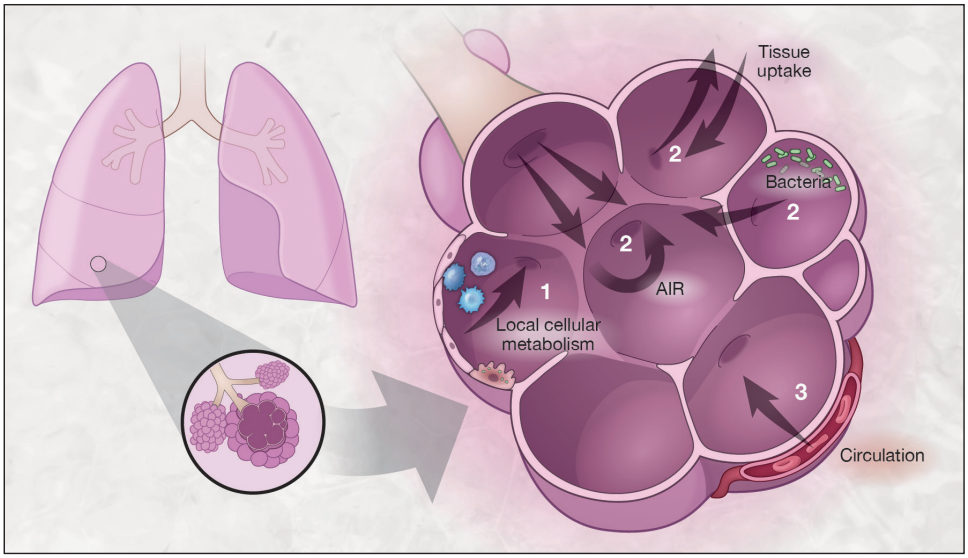

Figure 1. Systemic diagram of airways and alveolus with the various sources of exhaled VOCs.

Exhaled VOCs can originate from (1) endogenous VOCs from conducting airways and alveoli, (2) exogenous VOCs inhaled and subsequently exhaled or originating from the resident microbiome, or (3) systemic VOCs generated elsewhere in the body and transported to the lungs via the blood circulation. VOC, volatile organic compound.

VOC sampling and handling

The potential to noninvasively sample breath VOCs is core to the attractiveness of these biomarkers. Depending on the specific application, a variety of techniques to collect exhaled breath are currently available. Progress is made in investigating the influence of these various techniques on exhaled VOCs to provide suggestions for standardization [11–13]. Fortunately, the lack of international guidelines for sampling of VOCs is currently being

addressed by task forces. An overview of the core factors that are relevant with respect to collecting and handling exhaled breath are provided in table 2.

Table 1. Criteria for an ideal biomarker

1. Sensitive and specific for the diagnosis of the disease process
2. Reflect or be a very clear surrogate of the pathophysiological mechanism
3. Be stable and only vary with events known to relate to disease progression
4. Predict early-stage disease development
5. Predict disease progression
6. Be responsive to interventions known to be effective

Adapted from Stockley et al.[10]

Table 2. Key factors in breath collection and handling.[13]

Key factors	
Air sampling	Direct sampling Reusable collection bag with thorough cleaning Disposable collection bag
Air collection device	VOCs derived from collection device Disposable collection device Reusable collection device (cleaning agent VOCs)
Air collection method	Nasal/Oral sampling Tidal breathing vs forced exhalation Exhalation after breath hold Forced exhalation Flow
Environmental influences	Baseline samples of environmental air Wash-out period by inspiratory VOC filter
Storage	Direct analysis Storage on sorbent tubes for prolonged stabilization of VOCs Storage conditions

VOC, volatile organic compound.

VOC analysis techniques

The concepts of the analysis of volatile biomarkers can be understood best by discussing the two ends of the spectrum of available techniques (figure 2). On one hand, these encompass chemical analytical techniques allowing identification of specific compounds. At the other end of the spectrum are pattern-recognition-based techniques allowing probabilistic discrimination of biomarker profiles. It is, however, important to realize that many techniques share features with both of these basic concepts.

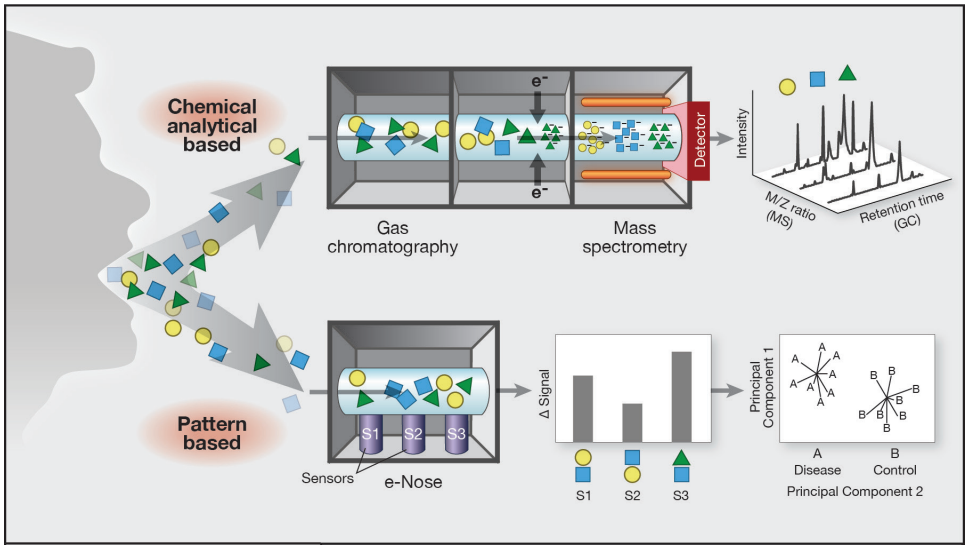

Figure 2. Analytical techniques for exhaled VOCs.

Two ends of the spectrum of VOC analysis techniques are depicted. Left : Exhaled VOCs are a complex mixture of different compounds (depicted by triangle, circle, and square symbols). The top of the image displays GC and MS as prototype chemical analytical technique allowing identification of individual components by comparing the mass to charge ratios of individual compounds with a previously established reference library. The bottom of the image displays the e-Nose as a prototype pattern recognition-based technology. e-Nose sensor are cross-reactive to multiple compounds in the VOC mixture, resulting in a pattern of sensor responses driven by, and characteristic of, the composite mixture of VOCs. These patterns can be compared with those previously encountered by pattern recognition algorithms to allow classification of individual cases. e-Nose, electronic nose; GC, gas chromatography; MS, mass spectrometry.

Chemical analytical techniques

Gas chromatography (GC) coupled to mass spectrometry (MS) is the current gold standard for the identification of individual chemical compounds [14]. GC-MS allows identification of individual compounds in a breath sample on the basis of their elution time from a capillary column and their mass to charge ratio. Clinical implementation of GC-MS is, however, relatively complex because of the requirement for highly trained personnel, the laborious analysis, and, therefore, the costly application. Recent years have, however, seen major advances in these techniques, such as the development of selected ion flow tube mass spectrometry, which is well suited for breath analysis because it allows real-time quantitative analysis of VOCs down to the parts per trillion range and is technically less demanding [15]. Furthermore, miniaturization of devices such as ion-mobility spectrometry may allow low-cost, on-site detection of specific compounds [16].

Pattern recognition-based sensors

At the other end of the spectrum are multiple-sensor devices resembling mammalian olfaction, therefore dubbed electronic noses (eNoses) [14]. VOCs competitively interact with cross-reactive sensors, allowing multiple VOCs to interact with the same sensor based on their affinity for both the sensor and its substrate. Likewise, multiple sensors interact with the same volatile. This is comparable to the powerful mammalian olfactory system and results in a pattern of firing sensors that is driven by, and characteristic of, the composite mixture of VOCs [17]. These patterns can be compared with those previously encountered by pattern recognition algorithms. Notably, this technique does not identify individual compounds but rather provides probabilistic recognition, which forms the basis of assessing diagnostic accuracy. eNose technology is relatively cheap, easy to use, and provides on-site results holding promise for its use as a point-of-care tool if properly validated and standardized [14].

CURRENT STATE OF VOC RESEARCH AND ITS FUTURE POTENTIAL

The key opportunities and critical challenges for the application of VOCs in clinic are illustrated by the collective experience of their use in three diseases: lung cancer, respiratory infections and obstructive lung disease.

Lung cancer: breath volatiles in screening

The noninvasive nature of VOC analysis makes it well suited for population-based screening for lung cancer. Studies to date using both GC-MS and eNose have shown a sensitivity and specificity ranging from 50-100% and 80-100%, respectively, for discrimination of healthy control subjects and patients with lung cancer *in vivo* [7, 18, 19]. Both research on *in vitro* tumor cell lines and *in vivo* breath analysis has aided in the identification of compounds driving these signals, which predominantly are hydrocarbons, alcohols, aldehydes, ketones, esters, nitriles and aromatic compounds [4, 20, 21]. Some of these markers have shown to decrease after resection of non-small cell lung carcinoma, providing further evidence they are linked to tumor presence [4].

It is important to realize that the exact origin of most of these VOCs is unknown. This means there is insufficient evidence that volatiles that discriminate between "gold-standard" healthy control subjects and carefully selected patients with lung cancer have sufficient accuracy to establish lung cancer in an intention-to-diagnose population. The latter can be characterized by comorbid conditions, such as smoking and COPD, potentially hampering the specificity of identified volatiles for lung cancer. This may be of even greater importance with respect to pattern-based techniques that do not identify individual compounds. It is, therefore, essential to apply VOC analysis with an *a priori* objective regarding the confirmation (high

specificity) and/or the exclusion (high sensitivity) of disease in the population in which the clinical application is intended [22]. Two small studies applying such principles showed that patients with malignant pleural mesothelioma could be discriminated from patients with benign asbestos-related disease and subjects with asbestos exposure with a reasonable accuracy of 80.8% [23, 24].

An appealing alternative to identify potential volatile biomarkers is that employed by the team of Hoassam Haick [25]. They identified mutation-specific volatiles by GC-MS analysis of headspace VOCs of tumor cell lineages with a known oncogenic K-Ras or epidermal growth factor receptor (EGFR) mutation. These were used to create a nanosensor array discriminating the cell lines with an accuracy of 84% to 96%. Although the specificity of these tailor- made sensors has to be determined *in vivo*, the concept of building sensors for disease-specific volatiles is appealing because it can boost the accuracy of breath-based tests and simultaneously improve knowledge of the origins of these VOCs.

By combining appropriately designed studies with technical expertise, the potential for breath-based diagnostics in lung cancer becomes realistic. For screening purposes, this requires excluding disease by maximizing sensitivity and negative predictive value enabling selection of patients for more elaborate testing. This will require large-scale prospective cohort studies to identify the suitable screening population, frequency of screening, and subsequent diagnostic approach, which will require many years of research. Developing such a breath-based low-cost, noninvasive screening test for an at-risk population may enable early detection and reduction of health-care costs with a better cost-effectiveness and risk profile than current screening programs.

Respiratory infections; point of care diagnostics

With respect to respiratory infections, the primary value for VOC-based analysis lies in early detection of infection both *in vitro* and *in vivo* and in monitoring of the host response [9, 26]. For TB, the development of a volatile biomarker test has many potential advantages over current diagnostic techniques because such a test could be low-cost and easy to implement in low-income countries [27]. *In vitro* analysis of the headspace of cultures revealed several TB-associated VOCs [28, 29]. Probabilistic analysis by eNose reached promising overall specificity and sensitivity of 91% and 89% respectively [30], although the accuracy in smear negative samples was only 69% [31]. The diagnostic potential of these *in vitro* volatiles can subsequently be assessed *in vivo* under the (unlikely) assumption that *in vivo* and *in vitro* metabolism of *Mycobacterium tuberculosis* is identical and that pathogen volatiles are not altered by the host. In fact, some studies did reproduce volatiles similar to those identified in cultures in the breath of subjects [32], whereas others did not [33]. By combining both host

response and pathogen derived VOCs, such *in vivo* studies reached accuracies of 79% till 85%, albeit not all in the intention-to-diagnose population [32–34].

Besides TB, volatile biomarkers have potential in diseases such as ventilator-associated pneumonia (VAP) [35] and cystic fibrosis (CF) [36]. Studying infections in CF, however, is exemplary of the extra level of complexity inherent to such studies because VOCs may relate to both the primary disease process and the infection. The presence of multiple simultaneous infections and nonspecificity of biomarkers to a single infection further complicates such analysis [37, 38]. The first proof-of-concept studies have shown patients with CF with and without *Pseudomonas* infection can be discriminated by a panel of VOCs [36]. Furthermore, children with and without exacerbations could be discriminated based on their VOC profile [39]. If detailed understanding of both host response and pathogen VOCs becomes available, this may ultimately change the way we diagnose infectious disease and monitor treatment response. Prior to effectuating such a promise, research will need to focus on *combining in vitro* and *in vivo* studies to identify key markers for specific pathogens, after which these can be validated in studies emulating clinical practice.

Obstructive lung diseases: phenotyping and monitoring of individual patients

The value of VOCs in obstructive lung diseases lies primarily in the monitoring and phenotyping of disease. The first proof-of-concept studies found 86% to 100% accuracy in discriminating healthy control subjects and gold standard patients with asthma [40, 41]. Fens and colleagues were able to discriminate patients with asthma and COPD by eNose and confirmed this discrimination by external validation (sensitivity 85%; specificity 90%) [42, 43]. Furthermore, a small study in children identified eight candidate asthma volatiles by GC-MS [44].

The largest unmet need in asthma and COPD is stratification of patients for antiinflammatory therapy with inhaled steroids or new biologicals, based on the inflammatory profile in the airways [45]. Several independent studies already linked VOC (profiles) in asthma and COPD to the inflammatory cell type in sputum and disease activity [46–48]. Indeed, steroid responsiveness in patients with asthma was shown to be predicted by eNose even with greater accuracy than by sputum eosinophils or exhaled nitric oxide [46]. Furthermore, patients with asthma exhaled increased levels of pentane during an exacerbation, decreasing to the level of control subjects when the exacerbation subsided [49]. In line with this, recent data suggest that VOCs allow discrimination of exacerbations in childhood asthma [50]. Especially in such heterogeneous diseases, individual daily monitoring and tailoring of therapy may empower patients to regain control of their disease and its therapy [51]. With respect to obstructive lung diseases, large multicenter trials such as U-BIOPRED (www.ubiopred.eu) validating these biomarkers are currently underway. These aim to provide evidence to determine whether and how clinical implementation of these techniques is beneficial to patient care.

Challenges and future directions

The potential benefits of VOC biomarkers are its noninvasiveness, speed, low-costs, and applicability in low-income countries. Much work, however, is needed before VOC-based diagnostic tools meet the criteria in table 1 and can be implemented, because current progress can only be classified as phase 2 to 4 on the 10-step technology readiness assessment scale [52].

A key issue with volatile biomarkers identified to date is the relative absence of independently reproduced biomarkers undermining the reliability of identified biomarkers. Part of these differences in established markers is likely due to the current lack of exchangeable collection techniques, devices and, data-analysis protocols. A European Respiratory Society task force is currently underway to provide guidelines for standardized methodology to maximize the compatibility of different datasets and minimize false discovery rate through stringent statistical approaches [53]. This furthermore requires a stepwise development and validation of omics-based biomarkers in clinical diagnosis and management, for which concrete criteria are available [54].

A second source of variation between breathomics studies are potential confounders. Indeed, factors known to influence VOCs include age, sex, pregnancy, medication, diet, and smoking, among many others [55–58]. In fact, even very mild exertion can change exhaled volatiles for hours [59, 60]. This knowledge is important for the interpretation of identified VOCs, but strict avoidance of all potential confounders is likely not worth pursuing. First, there is no evidence that dictating diet and minimizing exertion results in a more reproducible detection of biomarkers. Second, development of more specific and reliable sensor technology will reduce the effect of confounding VOCs on detecting target molecules. Foremost, however, extensive constraints on measurement protocols would render the technique impracticable, whereas its ease of use is central to its attractiveness.

A further constraint on the interpretation of established volatile markers is the fact that many of the predominant volatiles are related to a multitude of clinical conditions [61]. It is, therefore, unlikely that a disease can be characterized by a single biomarker. Fortunately, the multitude of VOCs enables the use of volatile biomarker profiles that may more robustly identify a clinical condition in the presence of confounding volatiles than a single biomarker [6]. This was illustrated by a study showing that prediction of steroid responsiveness in asthma was better achieved by the composite signal of exhaled VOCs measured by an electronic nose as compared with single biomarkers, such as sputum eosinophil count or exhaled nitric oxide [41].

With respect to sensor technology, the major advances can be expected from increased sensitivity and the development of sensors tailored toward detection of particular (classes of) VOCs and thereby specific conditions. Increasing sensor sensitivity is important, because the concentrations of some relevant VOCs have shown to be below the detection limit for certain sensors. Progress in this field is illustrated by promising developments lowering sensor detection limit and increasing selectivity [62]. This can either be achieved by selection of preassembled sensors through trial and error, potentially even without detailed knowledge of the target volatiles, or by building customized sensors that allow key-and-lock identification of disease-specific volatiles. Unfortunately, the relative lack of detailed knowledge on the core volatile markers in pulmonary disease has hampered the progress in sensor development relative to, for instance, acetone breath analysis in diabetes. Progress in this area can be reached by pooling of datasets and combining metabolomic measurements in tissue, blood, and breath. Furthermore, blocking specific enzymatic pathways and studying animal models may help to delineated exact pathways of VOC metabolism [63].

The adoption of breathomics in medical research and clinical practice would be greatly accelerated by the development of a so-called breath cloud, wherein exhaled breath VOC profiles could be stored alongside with anonymized clinical disease characteristics. This would allow real-time comparison of VOC patterns to provide an accurate diagnosis and phenotyping and will allow every subsequent measurement to contribute to the diagnostic algorithm, improving its accuracy while it is implemented in practice. This would furthermore allow a detailed comparison of the accuracy of VOC biomarkers with established biomarkers for that disease, which is essential to assess the value of such tests in clinical practice.

In conclusion, based on the current proof-of-concept data, clinical application of exhaled breath-based diagnostics and phenotyping is warranted, provided that the required (external) validation steps have adequately been taken [21]. To that end, breathomics needs to overcome key challenges such as focusing on the intention-to-diagnose population, using correct and standardized methodology, optimizing negative and/or positive predictive values, improving knowledge of VOC origins and developing tailored sensors. These milestones are all within reach when the field collectively follows the international guidelines on testing diagnostic accuracy and validation of composite biomarkers [22, 54].

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6

EXHALED MOLECULAR PROFILES IN THE ASSESSMENT OF CYSTIC FIBROSIS AND PRIMARY CILIARY DYSKINESIA

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ABSTRACT

Background

Early diagnosis and monitoring of disease activity are essential in cystic fibrosis (CF) and primary ciliary dyskinesia (PCD). We aimed to establish exhaled molecular profiles as the first step in assessing the potential of breath analysis.

Methods

Exhaled breath was analyzed by electronic nose in 25 children with CF, 25 with PCD and 23 controls. Principle component reduction and canonical discriminant analysis were used to construct internally cross-validated ROC curves.

Results

CF and PCD patients had significantly different breath profiles when compared to healthy controls (CF: sensitivity 84%, specificity 65%; PCD: sensitivity 88%, specificity 52%) and from each other (sensitivity 84%, specificity 60%). Patients with and without exacerbations had significantly different breath profiles (CF: sensitivity 89%, specificity 56%); PCD: sensitivity 100%, specificity 90%).

Conclusion

Exhaled molecular profiles significantly differ between patients with CF, PCD and controls. The eNose may have potential in disease monitoring based on the influence of exacerbations on the VOC-profile.

BACKGROUND

Cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) have a major impact on health and quality of life. Early diagnosis, frequent monitoring and vigorous treatment of respiratory infections are key to preserving lung function [1, 2]. For CF adequate screening methodology is available. PCD, however, remains challenging to diagnose as a single gold standard is lacking [1, 3]. Guidelines for the management of mucociliary diseases are based on the monitoring of symptoms [1, 4]. Unfortunately, the correlation between clinical symptoms and the underlying disease activity is generally poor and can only be established by invasive procedures [5]. Infection and inflammation may be present before clinical parameters change. Therefore, early detection and treatment of respiratory pathogens and exacerbations may improve clinical outcome. This creates the need for non-invasive methods in the monitoring of disease activity in CF and PCD.

Analysis of volatile biomarkers in exhaled breath has shown to provide an attractive method to monitor both infection [6] and inflammation[7–9]. These Volatile Organic Compounds (VOCs) are likely to originate from local and systemic metabolic processes reflecting underlying disease processes. Analysis of individual volatiles by Gas Chromatography – Mass Spectrometry (GC-MS) and measurement of nasal nitric oxide illustrated that concentrations of specific volatiles differ between CF patients and/or PCD patients and healthy controls [10, 11]. While GC-MS is an essential technique to link individual components to pathophysiological mechanisms its clinical applicability is hampered by the need for complex laboratory techniques and highly trained personnel. Alternatively, electronic nose (eNose) technology comprises arrays of promiscuous sensors interacting with the exhaled volatile mixture in its entirety, providing a so-called 'breathprint' by using pattern-recognition algorithms [12]. Biomedical application of eNoses is emerging [13] and various studies have demonstrated the value of this technology in the discrimination of patients with asthma [14, 15], COPD [8, 14, 15], lung cancer [16, 17] and ventilator-associated pneumonia [18].

For patients with CF and PCD exhaled breath analysis by electronic nose may allow both early screening and frequent monitoring, because the technique is portable, low cost and provides immediate results. According to START guidelines, the essential first step to assess in CF and PCD is to establish exhaled volatile profiles that discriminate a priori defined disease entities [19]. In this study we therefore hypothesized that patients with cystic fibrosis, primary ciliary dyskinesia and healthy controls have significantly different exhaled molecular profiles as determined by electronic nose. Furthermore we aim to study patients without an exacerbation to assess its potential influence on exhaled breath profiles. The secondary goal of this study was to examine whether significantly different breath profiles could be identified in patients with and without pulmonary exacerbations.

METHODS

Design

This study was designed as a cross-sectional case-control study. All patients performed a single study visit during which exhaled breath was analyzed and sputum or cough swabs were cultured. Spirometry was performed in patients six years and older by a trained lung function technician according to ATS/ERS guidelines [20].

Subjects

Children aged between 6 months and 18 years were included. By only including children we aimed to investigate CF and PCD patients that had relatively limited concomitant bacteria in their airways compared to adult patients. Patients were recruited during outpatient clinics of the VU University Medical Center Amsterdam, The Netherlands, between August 2011 and November 2011. Diagnosis of CF was based on clinical symptoms in combination with an abnormal sweat test (chloride > 60 mmol/l) and/or identification of mutations in both alleles of the CFTR-gene [21]. PCD diagnosis was based on a combination of clinical symptoms, evaluation of ciliary beat frequency and beat pattern by high-resolution, high-speed video microscopy and by transmission electron microscopy of ciliary ultrastructure as recommended by the European Respiratory Society Task Force consensus statement [1]. Furthermore, ciliary motility was also evaluated after cell culture to exclude secondary ciliary dyskinesia [22]. The presence of a pulmonary exacerbation in CF and PCD patients was determined after patients completed the study visit and was defined as the need to start additional antibiotic treatment as a consequence of a recent change in at least two of the following clinical parameters: change in sputum volume or color, increased cough, increased dyspnea, increased malaise, fatigue or lethargy, temperature over 38°, anorexia or weight loss, change in sinus discharge, change in physical findings on examination, decrease in pulmonary function by 10% or more and radiographic changes. This was done according to national CBO guidelines, based on internationally accepted criteria [5, 23, 24]. Healthy children were recruited during orthopedic outpatient clinics of the VU University Medical Center and the Academic Medical Center Amsterdam, The Netherlands. These two academic centers are situated within 10 km of each other and share the same patient population. Children were excluded in case of any pulmonary, inflammatory or metabolic disease. The study was approved by the Medical Ethical Committee of the participating centers. Written informed consent was obtained from parents and patients between 12 and 18 years. The study was registered in the Netherlands Trial Register, www.trialregister.nl under NTR 2847.

Measurements

Breath collection

Exhaled breath was collected using a modified spacer (Babyhaler, GlaxoSmithKline) with reverse valve system allowing tidal inspiration through a face mask and inspiratory VOC filter (A2, North Safety, Middelburg, The Netherlands) and tidal expiration into the spacer. The VOC filter minimizes the influence of environmental VOCs on the breath profile as a potential source of bias. The spacer was connected to the electronic nose during sampling for direct sample analysis during tidal breathing.

Electronic nose

We used a carbon black polymer based Cyranose 320 electronic nose (Smiths Detection, Pasadena, CA, USA). VOCs interact with the array of 32 polymer nanosensors to induce a fully reversible change in electrical resistance. The changes in resistance of all 32 sensors provide the raw data of the eNose and were combined by pattern recognition analysis into a so-called breathprint. This allows simultaneous analysis of the entire VOC profile instead of analyzing individual sensors which would only represent a limited fraction of the measured volatiles. The settings of the eNose used in this study did not provide immediate results to the investigator and patients. Raw eNose data were digitally imported into a study database.

Statistical analysis

The eNose data were analyzed by pattern-recognition algorithms. SPSS (version 16.0) was used for data analysis. Principal component reduction was used to capture the variance of the original breathprint into a set of orthogonal principle components (PCs), whereby reducing the dimensionality of the dataset to minimize the risk of overfitting [25]. Discriminating PCs were selected by unpaired *t*-test and subsequently used in a canonical discriminant analysis. The discriminant functions were used to construct a receiver operator characteristic (ROC) curve. The area under the curve (AUC) and optimum single spot test sensitivity and specificity were determined. This data was internally cross-validated by a bootstrapping procedure to minimize the risk of false positive findings according to current standards [25]. The current sample size was based on previous studies employing electronic noses in discriminating asthma [14, 15], COPD [15] and lung cancer [16] because no previous studies in CF and PCD were available.

RESULTS

Seventy-three subjects participated in this study, including 25 patients with CF (median age (yr), IQR, 11.4, 7.7-17.9), 25 patients with PCD (10.7, 7.1-14.5) and 23 healthy subjects (9.3, 5.4-12.6). The subject characteristics of the three groups are described in table 1. Median age and sex did not significantly differ between healthy controls recruited from the two outpatient clinics. Nine CF patients (36%) and 4 PCD patients (17%) had a pulmonary exacerbation. The number of positive sputum and cough swab cultures did not differ between patients with CF (16 out of 23 cultures) and PCD (8 out of 18 cultures). The presence of *Staphylococcus aureus* was significantly higher in cultures of CF patients as compared to cultures of PCD patients (p=0.04). *Haemophilus influenzae* occurred more frequently in cultures of PCD patients as compared to cultures of CF patients (p=0.02). No other significant differences in subject characteristics were found between CF and PCD patients.

Table 1. Clinical characteristics of the study population.

	Cystic Fibrosis	Primary Ciliary Dyskinesia	Healthy Subjects
Subjects (n)	25	25	23
Age (years)*	11.4 [7.7-17.9]	10.7 [7.1-14.5]	9.3 [5.4-12.6]
Male (n/total)	10/25	14/25	14/23
Best FEV_1 in past year (% predicted)*	92.0[81.5-111.0]	104.0 [80.5-109.8]	NA
Best FVC in past year (% predicted)*	99.0 [88.0-116.0]	110.5 [97.0-119.0]	NA
Pulmonary exacerbation (n/total)	9/25	4/23	NA
Positive bacterial cultures (n/total)	15/22	8/18	NA
S. aureus (n)	13[†]	2	NA
Pseudomonas spp.	5	1	NA
H. influenzae	1[§]	6	NA
S. maltophilia	1	0	NA
M. avium	1	0	NA
A. denitrificans	1	0	NA
M. catarrhalis	0	2	NA
S. pneumoniae	0	2	NA
Other	1	5	NA

* Data are presented as median and interquartile range. [†] p=0.04 as compared to PCD. [§] p=0.02 as compared to PCD. N, number; NA, not available; FEV_1, forced expiratory volume in 1 s pre-bronchodilator; FVC, forced vital capacity, pre-bronchodilator; *S. aureus, Staphylococcus aureus; Pseudomonas spp., Pseudomonas species; H. influenza, Haemophilus influenzae; S. maltophilia, Stenotrophomonas maltophilia; M. avium, Micobacterium avium; A. denitrificans, Achromobacter denitrificans; M. catarrhalis, Moraxella catarrhalis; S. Pneumoniae, Streptococcus pneumoniae.*

Breathprints from patients with CF (p=0.0004) and with PCD (p=0.0001) significantly differed from healthy subjects (figures 1A and 2A). The area under the receiver operator characteristic (ROC) curve (AUC) after internal cross-validation reached 0.76 (95% CI 0.62-0.90, sensitivity 84%, specificity 65%) and 0.80 (95%CI 0.67-0.93, sensitivity 88%, specificity 52%), respectively (figures 1B and 2B). Additionally, exhaled breath profiles differed significantly between patients with CF and PCD (p=0.001) (figure 3A). ROC analysis resulted in an AUC of 0.77 (95% CI 0.63-0.91, sensitivity 84%, specificity 60%) (figure 3B). CF (n=16) and PCD (n=19) breath profiles still differed significantly from one another after omitting breath profiles from patients with a pulmonary exacerbation (p=0.001). The AUC reached 0.77 (95% CI 0.60-0.95) and sensitivity and specificity increased to 95% and 63%, respectively. Detailed test characteristics for the diagnostic models are presented in table 2.

VOC profiles of CF patients (p=0.01) as well as PCD patients (p=0.01) with and without a pulmonary exacerbation differed significantly. ROC analysis resulted in an AUC of 0.76 (95% CI 0.58-0.95, sensitivity 89%, specificity 56%) and 0.90 (95% CI 0.76-1.00, sensitivity 100%, specificity 90%), respectively.

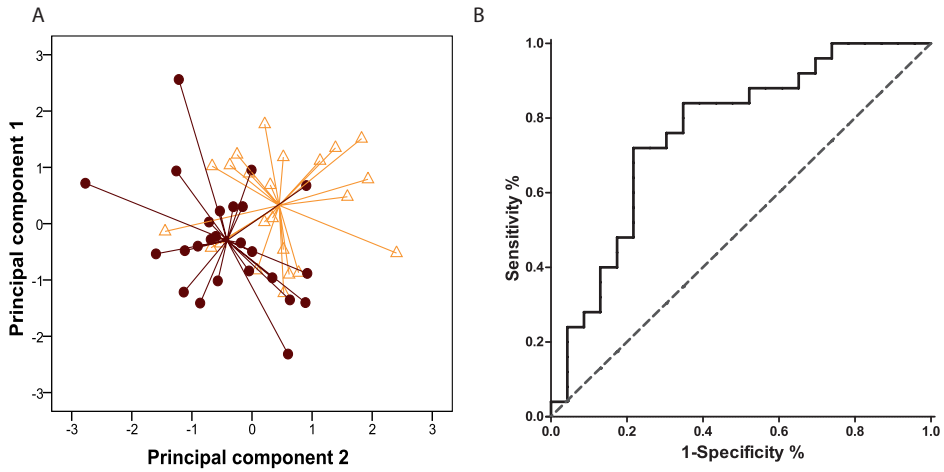

Figure 1. Discrimination of CF patients vs. healthy subjects.

(A) Two-dimensional principal component plot showing the discrimination of breathprints between patients with CF (circles) and healthy controls (triangles) along two discriminative principal components. P=0.0004. (B) Receiver operator characteristic (ROC) curve with line of identity of the breathprint discriminant function for the discrimination of CF patients and healthy controls (AUC 0.76).

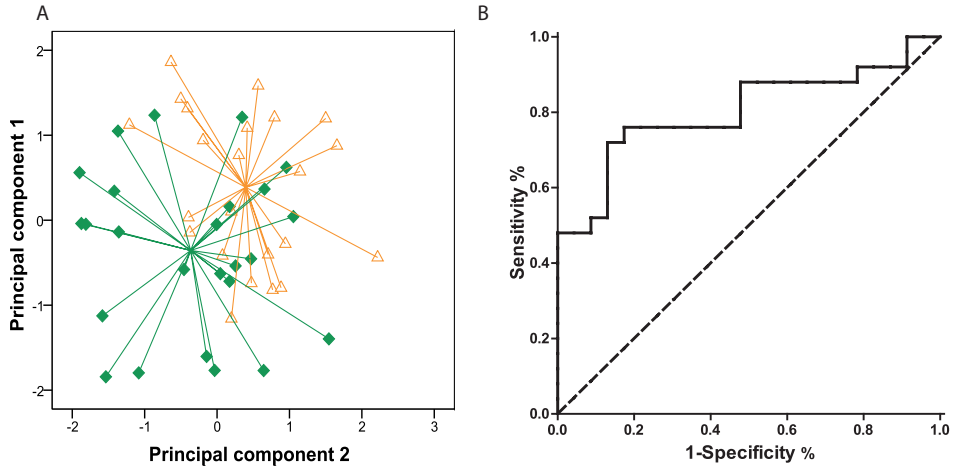

Figure 2. Discrimination of PCD patients vs. healthy subjects.

(A) Two-dimensional principal component plot showing the discrimination of breathprints between patients with PCD (diamonds) and healthy controls (triangles) along two discriminative principal components. P=0.0001. (B) Receiver operator characteristic (ROC) curve with line of identity of the breathprint discriminant function for the discrimination of PCD patients and healthy controls (AUC 0.80).

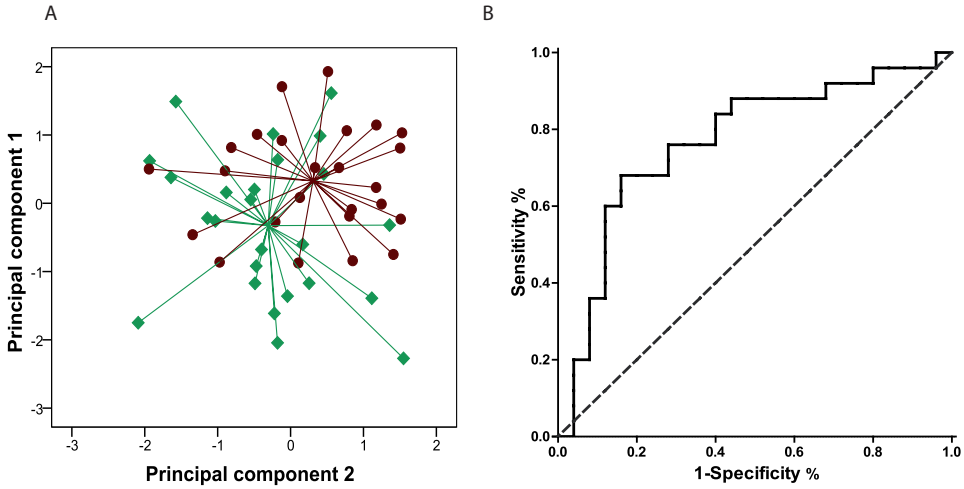

Figure 3. Discrimination of CF patients vs. PCD patients.

(A) Two-dimensional principal component plot showing the discrimination of breathprints between patients with CF (circles) and PCD (diamonds) along two discriminative principal components. P=0.001. (B) Receiver operator characteristic (ROC) curve with line of identity of the breathprint discriminant function for the discrimination of CF patients and PCD patients (AUC 0.77).

Table 2. Test characteristics of the receiver operator characteristic (ROC) curves for discrimination of CF patients, PCD patients and healthy subjects and for discrimination of patients with and without a pulmonary exacerbation, by exhaled breath analysis.

	N	AUC	95% CI	p-value	Sens (%)	Spec (%)
CF vs. healthy subjects	25 vs. 23	0.76	0.62-0.90	0.002	84	65
PCD vs. healthy subjects	25 vs. 23	0.80	0.67-0.93	<0.001	88	52
CF vs. PCD	25 vs. 25	0.77	0.63-0.91	0.001	84	60
CF pulmonary exacerbation vs. stable disease	9 vs. 16	0.76	0.58-0.95	0.031	89	56
PCD pulmonary exacerbation vs. stable disease	4 vs. 19	0.90	0.76-1.00	0.015	100	90
CF stable disease vs. PCD stable disease	16 vs. 19	0.77	0.60-0.95	0.006	95	63

Test characteristics of the receiver operator characteristic (ROC) curves for the discrimination of CF patients vs. healthy subjects, PCD patients vs. healthy subjects, PCD patients vs. CF patients and patients with and without a pulmonary exacerbation, by exhaled breath analysis.
CF, cystic fibrosis; PCD, primary ciliary dyskinesia; vs., versus; N, number; AUC, area under the curve; 95% CI, 95% confidence interval of the area under the curve; Sens, sensitivity; Spec, specificity.

DISCUSSION

Our study shows that CF and PCD patients have significantly different exhaled breath molecular profiles compared to healthy subjects as determined by electronic nose. Furthermore, we observed that CF and PCD have significantly different VOC profiles allowing separation with reasonable sensitivity but rather limited specificity. In addition, we observed that VOC profiles differed significantly depending on the presence of a pulmonary exacerbations. Our results suggest that exhaled breath analysis may have diagnostic and monitoring potential in mucociliary clearance diseases.

To our knowledge this is the first study using eNose technology in CF and PCD. Previously, Robroeks and colleagues identified individual VOCs related to CF by GC-MS analysis of exhaled breath [10]. Our findings extend these results by showing that children with CF have significantly different total VOC profiles compared to healthy subjects. The other novelty of this study was to show that the exhaled marker profile of PCD was significantly different from patients with CF and controls. Compared to nasal nitric oxide measurement in PCD the eNose has lower sensitivity and specificity for detecting PCD [11]. As with the eNose, although to a lower extent, nasal nitric oxide is not specific for PCD and shows overlap with CF, nasal polyps, chronic sinusitis and diffuse pan-bronchiolitis. Even though further studies are necessary eNose technique could be complementary to nasal nitric oxide in the diagnosis of PCD as it may be more easy to perform across all ages and may require less rigorous control of measurement circumstances.

In the present study, all included patients were well characterized according to internationally accepted guidelines, were recruited by the same operator and were derived from the same outpatient clinic [1, 21]. The control subjects were recruited from two outpatient clinics which were located in the same geographical area sharing the same patient population. Subanalysis showed that there was no significant difference between exhaled breath profiles between the healthy controls recruited at the different clinics (p=0.16). We aimed to study children in order to minimize the number of c respiratory infections as compared to adult patients. However, in 16 out of 23 CF patients and 8 out of 18 PCD patients, pathogens were still detected by culturing. It should be noted that negative culture results do not exclude the presence of airway pathogens, as various recent microbiome studies in CF have demonstrated [26]. Furthermore the number of positive bacterial cultures between CF and PCD patients in this study did not differ although the incidence of *S. aureus* and *H. influenzae* did. The presence of pathogens may therefore be a potential source of bias in the discrimination of CF/PCD from healthy controls. In a post-hoc analysis there was no significant difference between breath profiles in the presence or absence of an infection (p=0.33). The exact influence of pathogens on the VOC profile is however difficult to determine because the VOCs resulting from these infections are a combination of VOCs produced by the pathogen itself and the host response to that pathogen. A way of determining VOCs produced by individual pathogens is by studying the production of VOCs from *in vitro* cultures by GC-MS [13]. This however reaches beyond the scope of the current paper.

Even though CF and PCD are both characterized by chronic airway infection and neutrophilic inflammation, they originate from different pathophysiological mechanisms [5, 27]. It is likely that distinct inflammatory and metabolic processes generate partly different metabolites, explaining the different VOC mixtures in exhaled breath for both CF, PCD and healthy subjects [8].

As the determination of distinct breathprints in CF and PCD patients with a pulmonary exacerbation was our secondary objective, we reached a smaller sample size than for our primary objective. Therefore, the latter results are merely hypothesis-generating, requiring further validation in a larger cohort. Previous studies have shown that sputum of patients with PCD exhibit a more than 3-fold increase in interleukin (IL)-8 concentration, a trend towards a lower DNA concentration and lower levels of proteolytic enzymes as compared to subjects with CF, possibly reflecting differences in pathophysiological pathways [28, 29]. The significant differences between VOC profiles of these two mucociliary clearance diseases may thus very well originate from partially distinct inflammatory processes. It will require detailed characterization of individual VOCs by GC-MS to establish the predominant molecular compounds driving the signal. The latter is complementary to probabilistic diagnostic assessment by eNose.

Our study supports the notion that some of the discriminating VOCs are related to the host response by showing that CF and PCD breath profiles could be differentiated with slightly improved test sensitivity and specificity when patients with a pulmonary exacerbation were omitted. This suggests that disease specific VOCs for PCD and CF may exist. Furthermore this suggests that inflammatory changes can be detected by exhaled breath analysis potentially allowing monitoring of disease activity. It is important to investigate this in detail in a study specifically designed for this purpose as the current study was not sufficiently powered to address these questions. Therefore, the latter results are merely hypothesis-generating, requiring further study in a larger cohort.

Comparing gold standard diagnostic groups represents the first step of implementing a novel diagnostic technique into clinical practice according to the STARD guidelines [19]. Even though the currently observed sensitivities are promising, the specificities of the present analysis were still limited. We used bootstrapping procedures to minimize the possibility of false positive results which may occur when analyzing multivariate data [25].

Given the relatively high sensitivity for PCD in the present study, it can be inferred that breath analysis may have additional value in the initial diagnostic work-up of PCD. Particularly, since PCD is likely to be under diagnosed due to the lack of a single gold standard, ambiguous interpretation of diagnostic tests and the partial overlap of symptoms from healthy children suffering from recurrent respiratory tract infections [1, 30].

The observation that exhaled breath profiles differ significantly depending on the absence or presence of a pulmonary exacerbation suggests that the eNose may be a tool for non-invasive monitoring of disease activity in both CF and PCD. As recurrent respiratory infections and inflammation result in progressive lung damage, early detection and treatment are of major importance to improve clinical outcome in these patients. Future longitudinal studies should clarify whether VOC profiling of exhaled breath in CF and PCD patients may add to earlier identification and treatment of respiratory infections as established by quantitative PCR and microbiome technologies.

Furthermore the technological advances of breath analysis techniques are likely to increase its value for CF and PCD. The eNose device used in this study is based on the interaction of polymers with the VOCs inducing a resistance change. Many other techniques exist such as quartz microbalance sensors, metal oxide sensors and optical sensors. It's important to study the value of these sensors in the discrimination of CF and PCD in more detail.

New studies employing chemical analytical techniques such as GC–MS allow the identification of individual VOCs and may aid to connect VOCs to specific disease processes.

Importantly this can also facilitate the selection of the most suitable eNose sensors for the desired application and novel development of disease tailored sensors. This may greatly improve the clinical applicability of exhaled breath analysis by eNose.

In conclusion, our study showed that CF, PCD, healthy children and patients with and without pulmonary exacerbations have significantly different exhaled breath profiles as determined by an electronic nose. Therefore, after optimization and validation, exhaled breath analysis by eNose may eventually qualify as a non-invasive and easy to use tool in the monitoring of CF and PCD in clinical practice.

Conflict of interest statement

None of the authors have conflicts of interest to disclose.

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7

A RANDOMISED CONTROLLED TRIAL ON THE EFFECT OF INHALED HYPERTONIC SALINE ON QUALITY OF LIFE IN PRIMARY CILIARY DYSKINESIA

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ABSTRACT

Rationale

Hypertonic saline inhalation lowers airway mucous viscosity. Increased cough transportability may improve quality of life (QoL) in primary ciliary dyskinesia (PCD).

Methods

In this randomised controlled trial (RCT), PCD patients received twice-daily inhalations of hypertonic (7%) or isotonic (0.9%) saline for 12 weeks, with 4 weeks washout during crossover. Primary outcome was change in QoL measured by the St. George's Respiratory Questionnaire (SGRQ) total score. Secondary outcomes were SGRQ subscores, Quality of Life Questionnaire-Bronchiectasis (QoL-B) scores, lower respiratory tract infection symptoms, exacerbations, spirometry, systemic and sputum inflammatory markers, adherence and adverse events.

Results

There was no significant change in median (interquartile range) SGRQ total score between hypertonic saline (-2.6 (-9.0-1.5)) and isotonic saline (-0.3 (-8.1-6.1)) in 22 patients (age range 22-73 yr) (p=0.38). QoL-B Health Perception scale improved with hypertonic saline (p=0.03). Adverse events occurred more frequently with hypertonic saline, but were mild.

Conclusions

12 weeks of inhaled hypertonic saline did not improve SGRQ total score in adult PCD patients in this RCT, but the sample size was small. On the secondary and more disease-specific end-point of the QoL-B, a significant improvement was observed in the Health Perception scale. This study found little evidence to support the hypothesis that hypertonic saline improves QoL in PCD patients. We advise the use of disease-specific outcome measures in future trials.

RATIONALE

Mucociliary clearance (MCC) is a key part of the respiratory innate defence system, and is established by a combination of cough and synchronously beating epithelial cilia [1]. Genetic defects that cause dyskinetic or immotile cilia in primary ciliary dyskinesia (PCD) impair MCC [2]. The subsequent accumulation of mucus and susceptibility to inhaled pathogens causes recurrent airway infections, leading to chronic inflammation and airway remodeling. Daily symptoms such as cough and shortness of breath, but also pulmonary exacerbations and the progressive decline in lung function, significantly impact quality of life (QoL) in these patients [3, 4].

As there are no randomised clinical trials of PCD treatment, all recommendations are currently derived from cystic fibrosis (CF) guidelines and personal experiences [5, 6]. Despite the lack of available data, inhaled hypertonic saline is frequently prescribed to PCD patients to increase the ease of sputum expectoration, thereby improving MCC. In CF, these low-cost nebulisations have been shown to dramatically prolong the time to a new exacerbation and increase health-related QoL [7–9]. The rate of MCC is known to depend on coughing, ciliary beating, volume of the airway surface layer (ASL) and rheological properties of mucus [10]. Inhaled hypertonic saline has several mechanisms of action by which it improves MCC. First, it increases ASL volume. In CF, it rehydrates the depleted ASL, but even in healthy epithelium hypertonic saline causes an osmotic shift of water, increasing the ASL volume [8, 11]. Second, inhaled hypertonic saline lowers sputum viscosity in patients with CF and non-CF bronchiectasis, leading to a higher weight of expectorated sputum [12–14]. Third, it directly stimulates coughing [7, 15, 16]. Although results from *in vitro* and *in vivo* studies suggest that these effects are relatively short lasting, twice-daily nebulisations with hypertonic saline have shown clinical benefit in patients with CF and non-CF bronchiectasis [7, 8, 11, 17–19]. As epithelial cilia are dysfunctional in PCD, patients are largely dependent on coughing for their mucus clearance. As sputum rheology in PCD is similar to that in CF, lowering the viscosity may improve its transportability during coughing and regular physical activity [20–22].

Despite CF and PCD sharing many clinical characteristics, they are pathophysiologically distinct and patients may therefore respond differently to various treatments [23, 24]. This is exemplified by observations in a study with DNase treatment [25, 26]. Although not studied exclusively in PCD, but in a heterogeneous group of non-CF bronchiectasis patients, inhalations with DNAse were found to be harmful in this group while being highly effective in CF. Therefore, there is an urgent need to evaluate treatment options in PCD patients.

To explore the possible effect of inhaled hypertonic saline on QoL in adults with PCD, we performed a double-blind randomised controlled crossover trial. We chose the St. George's Respiratory Questionnaire (SGRQ) as our primary outcome as this is the most commonly used patient-reported outcome in non-CF bronchiectasis patients [27]. Additionally, we included the recently developed Quality of Life Questionnaire-Bronchiectasis (QoL-B), a more disease-specific patient-reported outcome, which was still under validation at the time this study was conducted [28]. We hypothesised that 12 weeks of twice-daily inhalations with hypertonic saline would improve health-related QoL in adult PCD patients compared with isotonic saline.

METHODS

Study design

A double-blind randomised controlled crossover trial was conducted over a 28-week period with 4 weeks washout between interventions (figure 1). Recruitment and study procedures took place between April 2014 and May 2015 at the VU University Medical Center and Academic Medical Center (Amsterdam, The Netherlands). The ethics committees of both centres approved the study and all participants gave written informed consent. The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki, and adhered to the CONSORT guidelines (online supplementary table S7.1) [29]. The trial was registered at the Netherlands Trial Register (www.trialregister.nl, trial number 4674), the Dutch primary registry which is recognised and acknowledged by the World Health Organization and International Committee of Medical Journal Editors.

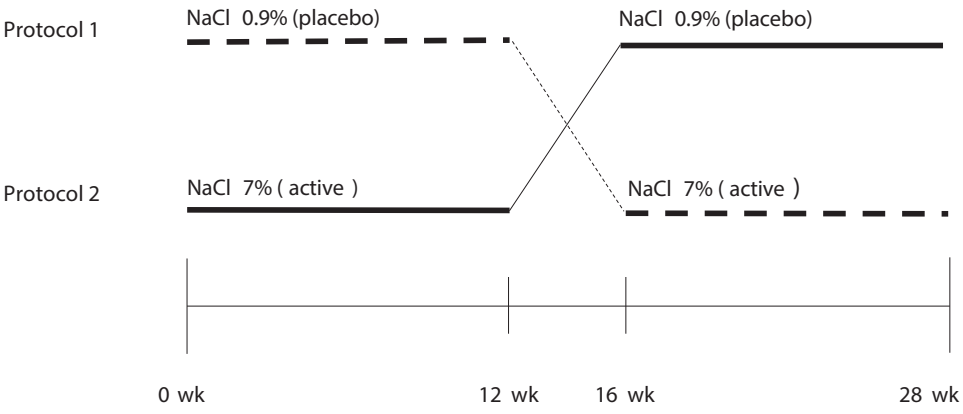

Figure 1. Crossover study design.

Participants and setting

Patients with a confirmed diagnosis of PCD, who were at least 18 years old and were in a clinically stable condition, were eligible to participate in the study. Patients were considered clinically stable when they had no pulmonary exacerbation in the previous 4 weeks and their forced expiratory volume in 1 s (FEV_1) was within 10% of the best value obtained during the previous 6 months. Detailed information on diagnostic, inclusion and exclusion criteria is available in the online supplementary material. Patients were informed about the study by their treating physician or by the national patient organisation. The investigators provided detailed study information and enrolled the patients.

Randomisation

A randomisation list for the order of treatment with two equal blocks was computer-generated and provided by an external pharmacy that prepared the study medication. Allocation concealment was handled by the pharmacies of the participating centres. The investigators, participants and treating physicians were unaware of the allocation throughout the study.

Interventions

Participants received twice-daily inhalations of 5 mL hypertonic (7%) saline and isotonic (0.9%) saline, with 0.25 mg/mL quinine sulphate added to mask the taste [7]. Trial medication was packed in glass ampoules and inhaled through a mouthpiece attached to a Porta-Neb side-stream nebuliser and compressor (Philips Respironics, Amsterdam, The Netherlands). 400 µg of a short-acting β-agonists bronchodilator was administered before inhalation of trial medication to prevent possible bronchoconstriction [7]. When patients did not tolerate a short-acting β-agonists, an anticholinergic was given. Standard care was maintained throughout the trial [5]. Concomitant therapies other than mucoactive treatments were approved and used at the discretion of the patient's physician.

Procedures and outcomes

After randomisation, patients underwent follow-up at week 6, 12, 16, 22 and 28 (figure 1). The primary end-point was change in SGRQ total score (0-100, with 100 being worst QoL) after 12 weeks of treatment [27]. Secondary end-points consisted of previously recommended treatment outcomes in PCD and established markers for monitoring lung function and sputum and systemic inflammation in chronic lung disease [5, 30]. These included subscores of the SGRQ and the QoL-B (0-100, with 0 being worst QoL), modified lower respiratory tract infection visual analogue scale (LRTI-VAS), pulmonary exacerbations, spirometry, serum C-reactive protein, erythrocyte sedimentation rate, white blood cell count and cell differentiation, microbiological evaluation, sputum cell differentiation, sputum neutrophil

elastase, interleukin-1β, -6, -8, and -10, tumour necrosis factor-α, myeloperoxidase, interferon (IFN)-α and -β, adherence, and adverse events [28, 31]. Adherence was determined by the investigator count of all ampoules. Additional information is available in the online supplementary material.

Statistical methods and sample size justification

Statistical analysis was performed on data from the intention-to-treat population and the per-protocol population (online supplementary material). The difference in change across the treatment and control phase was tested with the paired t-test or with the Wilcoxon signed rank test if data was not normally distributed. A linear mixed model, with intervention and visit number as fixed effects, was used to analyse the pattern of change across time. Log transformation was used if data was not normally distributed.

A 4-point reduction in SGRQ total score has previously been used as the minimal clinically important difference (MCID) [32]. We set the target sample size for this study to 24 patients. This was based on a combination a sample size calculations using the reported MCID and additional subjects for possible withdrawals. A minimum sample size of 14 patients was calculated to give a power of 0.80 in order to detect an improvement of 4 points in the SGRQ total score, estimating a standard deviation of 4 points, a within-patient correlation of 0.3 and a significance level of 0.05.

RESULTS

Study participants

Of the 86 patients screened for eligibility, 22 patients were included in the study and underwent randomisation (see the CONSORT flow diagram in figure 2). Online supplementary table S7.2 shows the reasons for declining study participation. The baseline characteristics of the participants entering the study are shown in table 1. Baseline values that were not significantly different at the start of the intervention periods were consistent with no carryover effect. Four participants discontinued the study. Three participants, of which two received hypertonic saline at the time, discontinued due to adverse events (chest tightness (n=2) and nausea (n=1)), which were possibly related to the trial medication. Another participant discontinued the study following hospitalisation for treatment of a subarachnoid haematoma, which was unrelated to the trial medication.

Primary outcome

Median (interquartile range (IQR)) change in SGRQ total score was -2.6 (-9.0- 1.5) after 12 weeks of twice-daily hypertonic saline inhalations *versus* -0.3 (-8.1- 6.1) after isotonic saline inhalations, which did not significantly differ from each other (p=0.38). The difference between these study periods did not reach the MCID of 4. Linear mixed model analysis did not show a significant effect of hypertonic saline treatment across time (p=0.57).

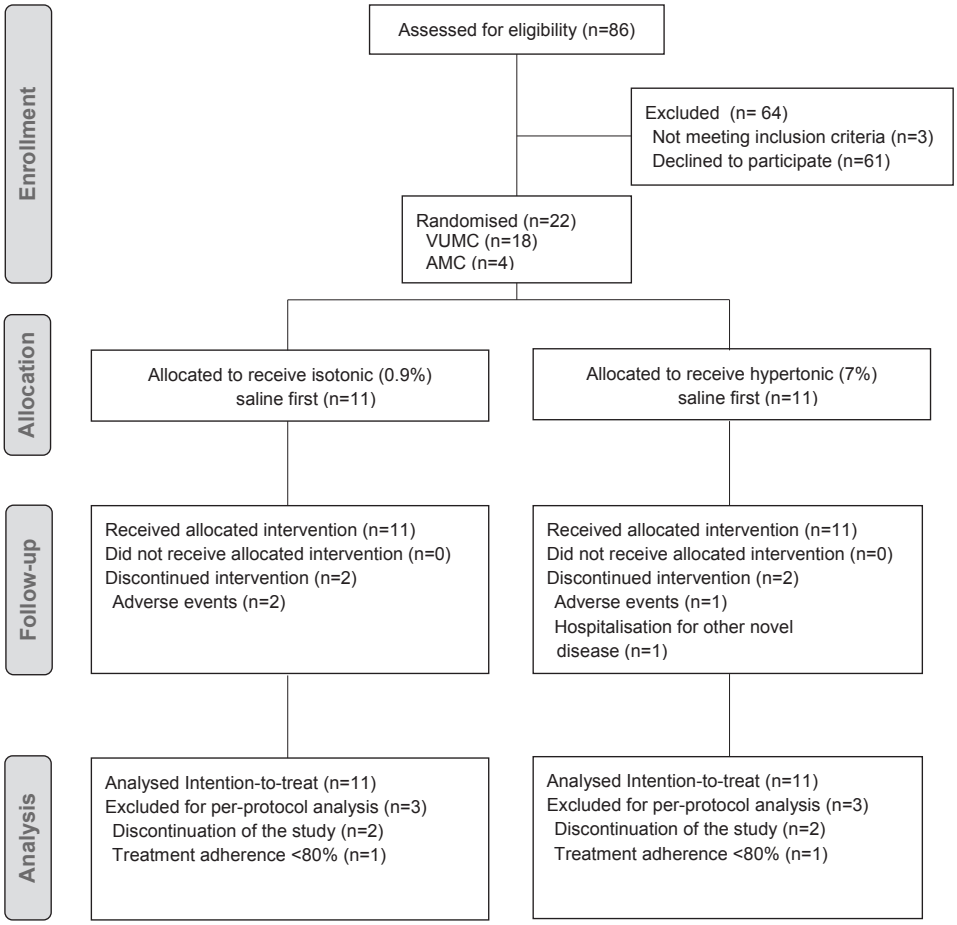

Figure 2. CONSORT flow chart summarising the progress of patients through the trial.
VUMC: VU University Medical Center; AMC: Academic Medical Center

Table 1. Baseline characteristics

Age years	47.6	[26.9-58.1]
Female	16	[72.7]
Body mass index kg/m²	25.8	[23.6-28.7]
FEV_1% pred	75.5	[58.8-94.5]
FVC % pred	92	[83.0-105.5]
FEF_{25-75}% pred	34.5	[25.3-65.0]
Chronic colonisation	10	
Haemophilus influenzae	7	
Staphylococcus aureus	2	
Streptococcus pneumoniae	1	[45.5%]
Achromobacter xylosoxidans	1	
Serratia marcescens	1	
Escherichia coli	1	
Medical Research Council dyspnoea scale score	2.0	[1.0-3.0]
Exacerbation frequency in last 12 months		
0-2	9	
≥3	13	
Bronchiectasis severity index score*	4.0	[2.0-7.0]
Mild	12	[54.5]
Moderate	7	[31.8]
Severe	3	[13.6]

Data are presented as a median (interquartile range), n (%) or n. FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; FEF_{25-75}: forced expiratory flow at 25-75% of FVC. *: the number of lobes affected by bronchiectasis, which was needed for calculating the bronchiectasis severity index, was based on high-resolution computed tomography images in 13 patients and on chest radiograph images in the other 9 patients.

Secondary outcomes

Median change in all health-related QoL scores are presented graphically in figures 3 and 4 for SGRQ and QoL-B, respectively. Online supplementary table S7.3 shows the secondary end-point data of SGRQ subscores, QoL-B scales, modifies LRTI-VAS scores, spirometry, systemic inflammatory markers, sputum cell differentiation and cytokines. No statistically significant improvement in all but the QoL-B Health Perception scale and the modified LRTI-VAS score for chest pain was seen after hypertonic saline treatment compared with isotonic saline. We observed a similar number of exacerbations in both intervention phases (median (IQR) 0 (0-1)) and no major confounding influence of exacerbations on the outcome parameters was observed. There were no differences in the clearance or acquisition of sputum bacteria. Online supplementary table S7.4 gives an overview of the responses patients gave to the open-ended question of the SGRQ in which they were asked to describe activities they feel most impaired in due to their disease.

QoL-B

The QoL-B Health Perception scale significantly improved after the hypertonic saline treatment phase compared with the control phase. Median (IQR) change was -8.3 (-16.7- 8.3) points in the control phase versus 8.3 (0.0- 10.4) points in the hypertonic saline treatment phase (p=0.03), exceeding the MCID of 8 points for this item. Linear mixed model analysis did not show a significantly different change across time between both interventions. Further, we observed clinically important improvements after hypertonic saline treatment in the QoL-B Respiratory Symptoms and Vitality scales, but they did not reach statistical significance. Change in Role Functioning was borderline significantly different between the treatment and control phase (p=0.05), but the effects size was below the MCID.

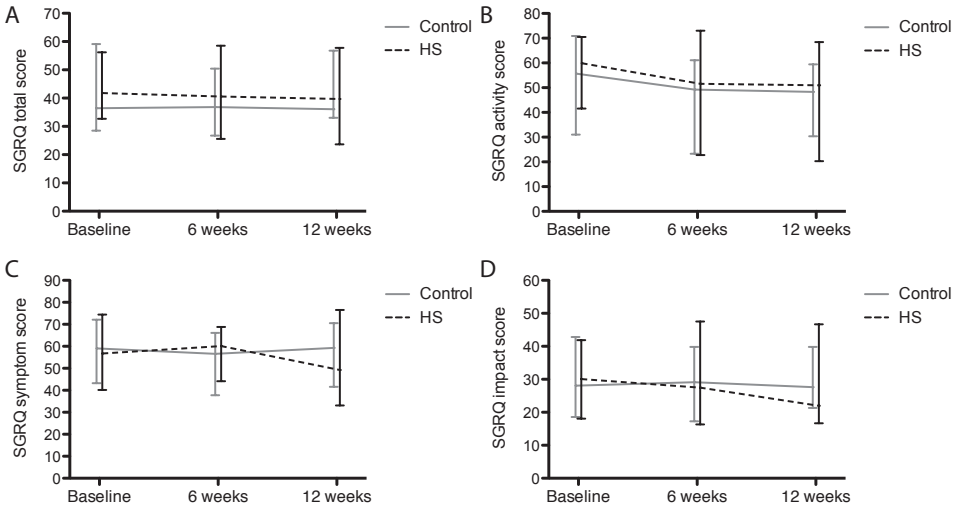

Figure 3. St George's Respiratory Questionnaire (SGRQ) scores with both interventions

(A) total score, (B) activity score, (C) symptom score, (D) impact score. Data are presented as median and interquartile range.

Modified LTRI-VAS

Modified LTRI-VAS score for chest pain improved slightly during hypertonic saline treatment phase with a median (IQR) change of -0.4 (-1.7- 0.15) compared with the control phase with a median (IQR) change of 0.0 (-0.4- 0.9) (p=0.03).

Adverse events

Adverse reactions to the study medication were seen in almost all patients, but occurred more frequently during the hypertonic saline treatment phase. Hypertonic saline most commonly caused oropharyngeal irritation, which generally subsided after a few days to

weeks (table 2). Three patients were admitted to the hospital during the study for treatment of a pulmonary exacerbation, a kidney transplant rejection and a deep venous thrombosis and for treatment of a subarachnoid haematoma. Only the latter occurred during the hypertonic saline treatment phase. None of these admissions were considered to be related to the trial medication.

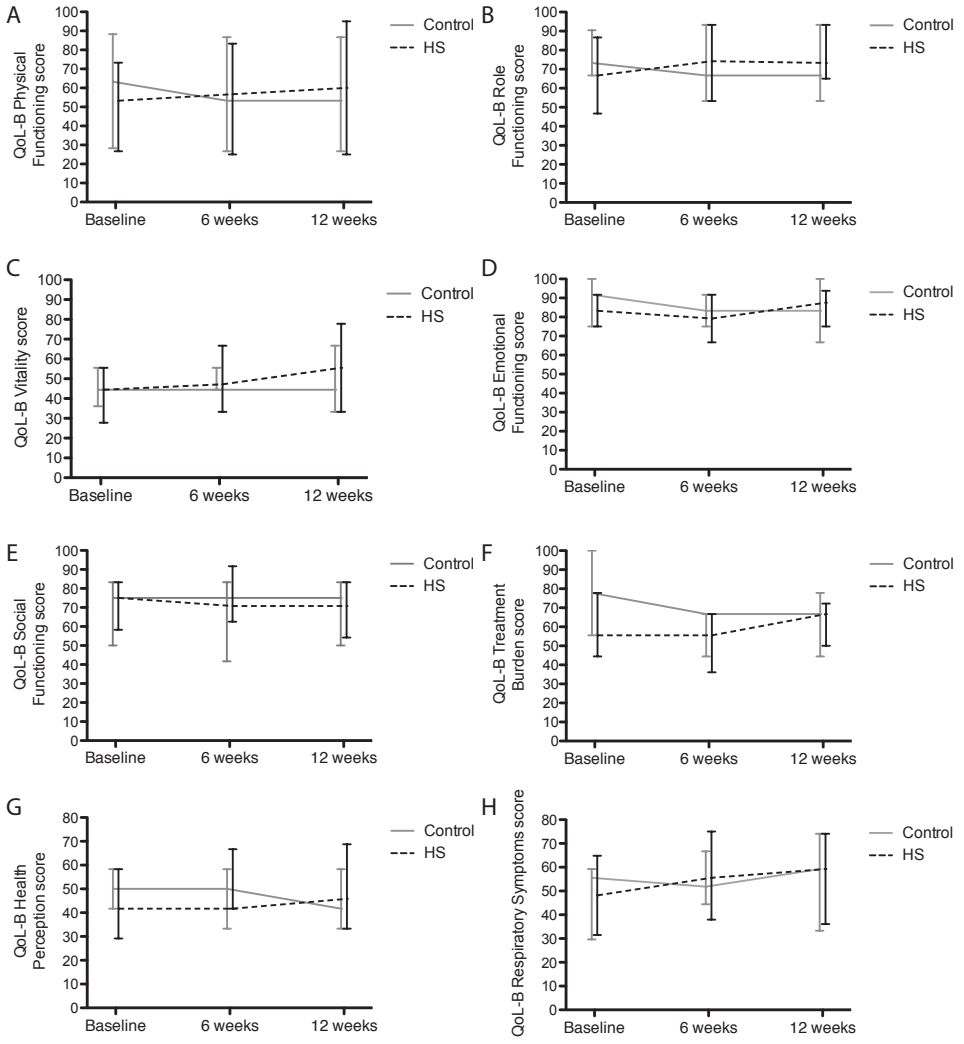

Figure 4. Quality of Life Questionnaire-Bronchiectasis (QoL-B) scale scores with both interventions
(A) Physical Functioning score, (B) Role Functioning score, (C) Vitality score, (D) Emotional Functioning score, (E) Social Functioning score, (F) Treatment Burden score, (G) Health Perception score, (H) Respiratory Symptoms score. HS: hypertonic (7%) saline. Data are presented as median and interquartile range.

Adverse events led to discontinuation of the study in three cases. Despite passing the test nebulisation, two subjects experienced symptoms which were suggestive of both bronchoconstriction and salbutamol intolerance. Another participant felt nauseated after each nebulisation with isotonic saline.

Table 2. Adverse events possibly related to study medication.

	Hypertonic (7%) saline	Control (isotonic (0.9%) saline)
Throat irritation/pain	11	4
Cough	9	3
Chest tightness	7	2
Increased sputum volume	4	3
Dizziness	4	1
Headache	2	2
Tiredness	2	1
Weight gain	2	1
Gastro-intestinal symptoms	0	2
Haemoptysis	1	0
Tingling sensation hands/chest	1	0
Hoarseness	0	1
Bad taste in mouth	0	1

Data are presented as n.

Adherence

Median (IQR) adherence to treatment, as judged by the number of returned ampoules from patients completing the study, was 93.4% (87.6-100%) for the hypertonic saline treatment phase and 93.0% (69.5-98%) for the control phase (p=0.21). Only two participants that completed the study had <80% compliance in both treatment phases.

Per-protocol analysis

Per-protocol analysis of patients completing the study and receiving at least a mean of 80% of trial medication in both treatment and control phase generally had no substantial effect on the effect size and the level of significance of the outcome parameters.

Observed mean differences between the hypertonic saline and control group, observed standard deviations and within-subject variation are provided for the primary and secondary outcomes in online supplementary table S7.5.

DISCUSSION

In this randomised controlled trial (RCT) we explored the effect of hypertonic saline on health-related QoL in adult PCD patients. 12 weeks of twice-daily inhalations with hypertonic saline did not significantly improve SGRQ total score in adult PCD patients. Hypertonic saline treatment did improve their health perception, as measured by the QoL-B questionnaire. In addition, a small but significant improvement in chest pain was observed, as determined by the modified LTRI-VAS. Participants generally reported low health-related QoL at baseline, similar to chronic obstructive pulmonary disease (COPD) Global Initiative for Chronic Obstructive Lung Disease grade C-D patients, but with relatively high interpatient variability depicting the clinical heterogeneity of the disease [33].

There are several possible explanations for the lack of a statistically significant difference between SGRQ scores of patients treated with hypertonic saline and isotonic saline. First, the observed difference in change of SGRQ scores after hypertonic saline treatment was smaller than the MCID, and the intersubject variability was larger than estimated. Consequently, the sample size was too small to detect these changes. In order to make informed sample size calculations in future larger trials we added detailed information on the observed differences and the variability (online supplementary table S7.5). Second, hypertonic saline may not improve QoL in PCD patients as it does in CF. In CF the ASL is dehydrated due to depletion of salt. In PCD and other non-CF bronchiectasis, where there is no problem with CF transmembrane conductance regulatory function causing electrolyte shifts but mucus is viscous due to inflammatory cells and cell debris, hydration may not have the same effect [20]. However, when sputum from CF and non-CF bronchiectasis patients was incubated with saline and placed on bovine trachea, a comparable dose-dependent improvement in transportability was observed [34]. In contrast to this animal model and other causes of bronchiectasis, epithelial cilia in PCD are dysfunctional and a lower viscosity will not lead to improved ciliary clearance in these patients. However, hypertonic saline has also shown to improve cough clearability [14]. Coughing, the primary clearance mechanism in PCD, has shown to remove a similar fraction of inhaled particles in PCD patients as in healthy nonsmoking subjects, but it does take more time to achieve [22]. Third, SGRQ total score may not have been sensitive enough to detect a clinical benefit in PCD patients. The scores for QoL-B Health Perception, Respiratory Symptoms and Vitality did show improvements that exceeded the MCID after hypertonic saline treatment, although only the first reached statistical significance. Naturally, these results need to be interpreted with caution as we did not correct for multiple testing in this first explorative study. In contrast to the SGRQ, the QoL-B is specifically designed to asses respiratory symptoms in bronchiectasis patients instead of COPD patients. It includes important symptoms that are very common in PCD,

e.g. "the feeling of a full chest", which are not addressed in the SGRQ. Furthermore, the QoL-B was developed with US Food and Drug Administration patient-reported outcome guidance, has fewer items, and has a shorter and less variable recall. This may suggest that it is important to use a disease-specific patient-reported outcome as primary outcome in RCTs to fully address the issues PCD patients face. A questionnaire encompassing all PCD-specific symptoms (PCD-QoL), including items on ear, nose and throat (ENT), was recently developed as part of the international FP7 BESTCILIA programme [35]. Hypertonic saline treatment is not expected to relieve ENT symptoms when delivered by a mouthpiece, but the use of a face mask may be beneficial. This also holds true for interventions with a more systemic effect [36].

In contrast to this study, Nicolson et al. showed improved SGRQ scores and spirometric values in non-CF bronchiectasis patients after 12 months of treatment [17]. However, these improvements were observed in both the treatment and control group, suggesting that hypertonic saline and isotonic saline may have similar beneficial effects or that patients benefited from participation in the trial, including the frequent check-ups. There is some evidence in COPD that isotonic saline could also have a positive clinical effect by humidifying the airway surface liquid [37]. However, the effect of nebulised saline is strongly dose dependent and isotonic saline was not observed to be of clinical benefit in the large CF trials [38–40]. In our study we also did not see any improvement in SGRQ total score following inhalations with isotonic saline, but sample size was small. In contrast to the present study and Nicolson et al., Kellett et al. did observe superiority of hypertonic saline over isotonic saline in QoL and exacerbation rate after only 3 months of treatment [17, 18]. However, with the relatively low exacerbation rate that we observed one would need a longer study duration to address this. Additionally, our study is the only one using an appropriate taste-masking agent, as was also done in the CF trials [7, 8]. The lack of masking the distinct taste of hypertonic saline may have caused inadequate blinding. Further, both the Nicolson et al. and Kellett et al. studies were conducted in non-CF bronchiectasis patients, whereas this study was done in PCD patients only [17, 18]. Non-CF bronchiectasis comprises a heterogeneous group of patients with bronchiectasis of various origins, including post-infectious bronchiectasis, immune deficiencies, autoimmune diseases, PCD and a large group of unknown aetiology. Bronchiectasis of different aetiology may respond differently to treatment. In addition, not all our patients had bronchiectasis.

The absence of a clinically important effect of hypertonic saline treatment on spirometry that we observed is in line with the Nicolson et al. study, but not with the Kellett et al. study [17, 18]. A reason for this could be the relatively good lung function of patients in the current and Nicolson et al. study, compared with the Kellett et al. study (FEV_1 % pred 75-85% versus 66%, respectively) [41].

The present study is the first to report longitudinal data on airway inflammation in PCD patients. Similar to previous cross-sectional studies in PCD, we found severe neutrophil-dominated inflammation and high levels of pro-inflammatory cytokines [20, 42–44]. We did not observe any change in sputum inflammatory markers after hypertonic saline treatment in our cohort, which is in line with data previously obtained in CF patients [7]. Our results extend these findings by also showing remarkably high levels of IFN-α and -β. While the role of these type 1 IFNs in antiviral innate immunity is well established, evidence about their possible ambivalent role in bacterial infections has been accumulating in the past few years [45].

We observed a relatively low participation rate of 26.5% in PCD patients that were eligible to participate in this study. Interestingly, the two main reasons for declining participation were either 1) too time consuming (hospital too far away or too much time spent on study medication and study visits) or 2) that they were not willing to stop and washout their hypertonic saline treatment that they were currently using. The first reason could be partially specifically related to the Netherlands, as inhabitants are not used to travelling long distances to a hospital and study procedures were only performed in the two academic hospitals in Amsterdam. We are not exactly sure how to avoid the second issue in future trials, although the prescribing rate of hypertonic saline in PCD patients may be lower in other countries.

In summary, this double-blind RCT demonstrated no significant effect of hypertonic saline in adult PCD patients on the primary outcome, *i.e.* SGRQ total score. We did observe a significant improvement of the Health Perception scale of the more-disease specific QoL-B and a small but significant improvement in the feeling of chest pain, measured by the modified LTRI-VAS. Based on these results, we advise that a disease-specific patient-reported outcome is used in future studies.

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8

GENERAL DISCUSSION

In this thesis we studied the genetic background of Dutch primary ciliary dyskinesia (PCD) patients. In addition, we studied the potential of exhaled breath analysis and hypertonic saline inhalations in the respiratory management of PCD patients. In this chapter we reflect on our main results and discuss methodological considerations. Finally, we address our recommendations and propose directions for future research.

PART I GENETIC DIAGNOSIS OF PCD

1.1 Unravelling the genetic background of PCD

Although PCD was first described in 1933, it took until 1999 to identify the first causative gene by a candidate gene approach [1, 2]. *Chlamydomonas* flagellar mutants carrying a defect in the *IC78* gene were observed to have similar axonemal ultrastructural abnormality as some PCD patients [2]. By isolating and sequencing the human homologue *DNAI1* in two siblings with PCD, two compound heterozygous loss of function mutations were found. The candidate gene strategy was adopted by others, resulting in the identification of many other genes related to outer dynein arm (ODA) or combined ODA and inner dynein arm (IDA) defects [3–5]. In addition, large consanguineous families with PCD were found in which homozygosity mapping, a form of linkage analysis, could successfully be applied. Using this approach in a large Lebanese family, including 4 affected individuals, identified the genomic region in which candidate gene *DNAH5* was present. *DNAH5* is now recognized as the most important PCD-related gene in which up to 30% of patients harbor mutations [6]. In contrast to this rather slow pace of PCD gene discovery by the candidate gene approach and linkage analysis, the development of next-generation sequencing (NGS) has led to a dramatic acceleration [7]. This technology enables sequencing of the entire genome of a single person, thus allowing identification of mutations specific to that individual. Since the completion of the initial sequencing of a human genome (i.e. the Human Genome Project) the discovery of the molecular basis of Mendelian disorders has more than tripled from 1,000 to 3,600, which accounts for about 50% of all Mendelian disorders described [8] (figure 1).

Since the widespread application of this technique in PCD individuals, more than half of the PCD-related genes have been identified in the last 5 years [7]. These findings majorly contributed to our knowledge on cilia biogenesis and function. Linking these genes to PCD gave more insight into their role in cytoplasmic pre-assembly of axonemal components, transfer into the cilium and intra-axonemal transport and attachment [7, 9]. This thesis describes the contribution of 2 novel PCD-related genes and the first genetic characterization of 74 Dutch PCD patients.

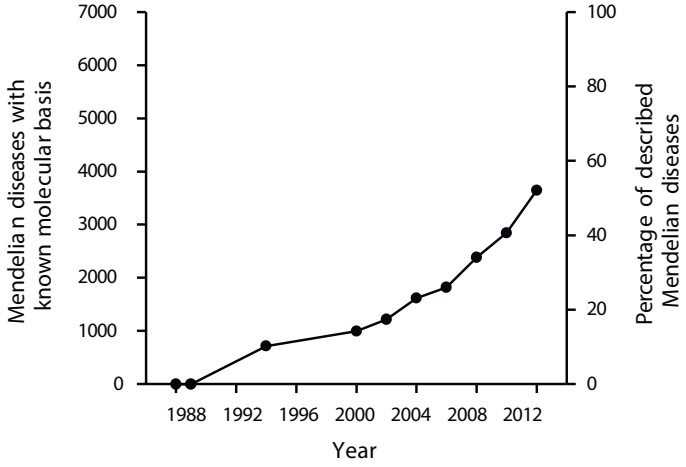

Figure 1. Mendelian diseases of known molecular basis [8].

In **chapter 2** we first show the capability of NGS to identify a founder mutation in the consanguineous population of Volendam, where linkage analysis failed to do in the past. Volendam is a small genetically isolated Dutch village in North Holland owing to religious and geographical reasons dating back to the 15th century. The consequence of genetic isolation is that individuals have a different genetic make-up than the rest of the population. A group of settlers bring only a small sample of the overall genetic diversity with them. As the population expands the limited gene pool results in inbreeding and eventually consanguinity. In Volendam this has led to a high prevalence of several diseases such as pontocerebellar hypoplasia type 2, fetal akinesia deformation sequence, rhizomelic chondrodysplasia punctata type 1, osteogenesis imperfecta type IIB/III and PCD [10] (**chapter 2**). In the past, linkage analysis has been used to try to identify the causative gene for PCD in Volendam inhabitants. Linkage analysis is based on the tendency of alleles that are close together on a chromosome to be inherited together during meiosis [11]. DNA from both affected and unaffected individuals are genotyped for polymorphic markers spread throughout the genome. One can then identify a chromosomal region that shows segregation of a disease-associated haplotype in affected individuals, and of a non-disease-associated haplotype in unaffected individuals. In **chapter 2** we describe the observation that the haplotype that is shared by individuals with PCD originating from the town of Volendam is only ~2 Mb in size. This could explain why a single locus was missed in past linkage mapping. By analyzing whole-exome sequencing data from only two individuals with PCD from Volendam we identified a novel homozygous mutation in *CCDC114*, an essential ciliary protein required for microtubular attachment of ODAs in the axoneme. All 16 PCD patients originating from Volendam that participated in our study

harbored the same pathogenic mutation in a homozygous manner. We demonstrated that this mutation is likely to date back from more than 70 generations and was thus brought into the Volendam population by two or more settlers that were carriers of the mutation. Further, we show that there is an increased incidence of PCD of about 1:400 newborns in Volendam, corresponding to a carrier frequency of the *CCDC114* mutation of 1:10 Volendam inhabitants. This is in stark contrast to the estimated incidence of PCD of ~ 1:10.000-1:30.000 in the rest of the world. The clinical impact of this study is huge as the founder mutation presents an important diagnostic target in this isolated Dutch Volendam population. It has enabled low cost Sanger sequencing of only one exon of the *CCDC114* gene in patients with clinical symptoms of PCD originating from Volendam, instead of the elaborate diagnostic evaluation that is usually required to diagnose PCD (**chapter 1)**. Further, carrier screening has become available for couples with affected relatives. As ~6% of PCD patients have a congenital heart defect, preconception carrier screening may optimize prenatal and postnatal care for these children. Similarly, founder mutations have been identified in American and Polish subpopulations with an increased incidence of PCD [12]. Identification of these mutations enables a simple diagnostic test for a large group of individuals with an increased risk of PCD. Although in American Amish and Mennonite communities that are geographically more dispersed, mutations in a few other genes were found in addition to a *DNAH5* founder mutation [12].

In **chapter 3** we describe the first observation of a recessive x-linked inheritance mode in four male PCD patients from 2 families, without syndromic co-segregation. In the vast majority of cases, PCD is inherited in an autosomal recessive mode. There are only rarely families described in which an autosomal dominant inheritance or recessive x-linked inheritance was observed [13–15]. These PCD-affected individuals additionally suffered from retinitis pigmentosa or had severe mental disability, which in both cases was the initial reason for the genetic investigation. We show that a hemizygous loss of function mutation in the x-linked *PIH1D3* gene causes PCD in males without another co-segregating disease. This is of major importance to the analysis of exome sequencing data, as the presumed inheritance pattern in PCD is autosomal recessive. Mutations in *PIH1D3* can therefore be easily overlooked, especially in male cases with ODA/IDA defects, without (I) syndromic co-segregation, (II) knowledge of the entire pedigree, (III) or without siblings or with only female siblings. Additionally, this inheritance pattern requires different genetic counseling on the risk of disease in the offspring of affected individuals than an autosomal recessive inheritance pattern. Shortly after the publication of our study it was suggested that *PIH1D3* mutations may be a prominent cause of X-linked PCD [16]. Olcese and co-workers identified pathogenic mutations in *PIH1D3* in 9,5% of affected males with PCD with a lack of dynein arms and so far unexplained genetic base of the disease. Therefore we suggest to

prioritize screening the *PIH1D3* gene in male cases with combined IDA/ODA defects and an unknown or suggestive X-linked inheritance pattern.

In **chapter 4** we characterized the gene defects in a Dutch PCD cohort. Data from this study confirm international observations that the majority of mutations in PCD are found in a rather small subset of genes and the others are relatively private mutations, occurring in a few families or in larger consanguineous populations. We do, however, observe a unique distribution of gene defects in the Dutch PCD population. *CCDC114*, *DNAI1* and *HYDIN* mutations seem much more prevalent in the Netherlands than in other countries. In case of *CCDC114* and *DNAI1* this is caused by founder mutations (**chapter 2 and 4**). In case of *HYDIN*, mutations may partially be overlooked in other cohorts, as data analysis is complicated by a pseudogene spanning most of the *HYDIN* gene exons [17]. Data analysis of next-generation sequencing results can lead to false negatives by interference of such pseudogenes. This occurs in cases where sequencing reads that can be mapped on two locations are automatically discarded by a software pipeline. Consequently, point mutations present in these sequences can be missed. This was illustrated by the identification of additional *HYDIN* mutations in two patients after re-mapping our raw sequencing data to the reference genome excluding the pseudogene (**chapter 4**). We propose that this method can also be used to improve exome sequencing data analysis of other genes that have pseudogenes. So far, over 11,000 pseudogenes have been annotated in the human genome [18]. On the other hand, ambivalent mapping of sequencing reads due to high sequence similarity often results in false positives. Therefore, we confirmed the location of all pathogenic *HYDIN* mutations we found in our patients with *HYDIN*-specific primers (**chapter 4**).

1.2 The role of genetic testing in the diagnosis of PCD

As more and more genes are linked to PCD we need to establish the role of genetic testing in the diagnostic approach [19]. As we describe in more detail in **chapter 1**, the diagnosis of PCD has been primarily based on evaluation of the ciliary ultrastructure by electron microscopy and the ciliary motion, by high-speed videomicroscopy. As a result of the discovery of many PCD-related genes in the past few years, recent guidelines have included genetic testing as an option in the diagnostic work-up of suspected PCD patients [19]. A genetic test may have many advantages over the currently available screening and diagnostic methods. A genetic test may improve the time to diagnosis, facilitates genetic counseling of families and improve our knowledge on genotype-phenotype relationships. **In chapter 4** we attempted to take a first step in the evaluation of the exact role of genetic testing in the diagnostic work-up of Dutch PCD patients. We aimed to determine the diagnostic yield of a targeted-exome gene panel in 74 pediatric and adult PCD patients. The target included 26 PCD-related genes that were identified at that time and an additional 284 candidate genes. We observed pathogenic

homozygous or compound heterozygous mutations in 68,9% of PCD patients. This yield would have been higher if it included the Volendam PCD patients (**chapter 2**) and if the panel would include 10 other genes that are currently also linked to PCD. In comparison, two other groups observed a diagnostic yield of 42-67% by using gene panels including 12-24 PCD-related genes [20–22]. These results underline that a targeted gene panel cannot be used as a sole test in the diagnosis of PCD. There may be several ways to increase the performance of a genetic test. By subsequently applying WES in patients without bi-allelic mutations, Marshall et al. increased the obtained diagnostic yield with ~15% [21]. This was improved with another ~8.8% by identifying copy number variations (CNV, i.e. large deletions or duplications) in patients with a pathogenic mono-allelic mutation [21, 23]. Although WES includes much more information about our DNA than a targeted-exome gene panel, it has the advantage of not showing unwanted findings, lower costs and a better reading depth, which decreases false negative and false positive findings.
Fortunately, recent technological advances are improving many of these aspects in WES. The huge advantage of using WES directly in all patients is that there is no need for re-sequencing if a targeted panel did not identify pathogenic mutations. This is illustrated by the relatively large group of PCD patients in our cohort in which we could not identify bi-allelic pathogenic mutations.

As an alternative to analyzing all WES data immediately, an in silico gene panel can be applied, including genes that are related to PCD specifically or to motile cilia in general. In **chapter 4** we give an example of the latter. We hypothesized that genes that are important in ciliogenesis share a common gene expression profile with significant upregulation during *in vitro* ciliogenesis in cell cultures of human airway epithelium. All but one of the PCD-related genes that are currently identified fell into a cluster of ~ 5500 genes that showed upregulation during ciliogenesis. Although this is still a large cluster of genes, using it as an in silico panel following WES could have several advantages. First, the a-priori chance of identifying mutations in a novel PCD-related gene is increased as the genes in this cluster have a similar expression pattern as currently known PCD-related genes. Second, the number of variants to analyze would be reduced to ~21% of total and third, it would reduce the chance of unexpected findings.

When using WES in the diagnosis of Mendelian disorders we need to bear in mind that it still has some technical challenges to face. First, WES can show a very uneven distribution of the sequencing depth. This can lead to many "uncovered" parts of the exome with a low reading depth that may include pathogenic mutations. The principal factors underlying this problem are related to the capture and PCR-amplification steps required for the preparation of sequencing libraries for WES. Second, the heterogeneous coverage prevents WES from

reliably detecting CNVs (i.e. large deletions or duplications). Third, WES only sequences the protein-coding part of the genome (~1%). Previously the rest of the DNA was considered "bulk", but there is evidence that over 70% of the genome is transcribed. Many of these transcripts do not code for proteins but have a regulatory role in gene expression, which can have pathological results when mutated. A universal challenge in all these approaches is to identify the functional relevance of novel sequence variants and to determine whether they can be responsible for the patients' disease (**chapters 2-4**).

1.3 Genotype-phenotype

Genotype-phenotype relationships can be straightforward in case a single mutation leads to a particular phenotype, but they are often more complex. This complexity arises from differences in penetrance and expressivity, which can be affected by modifier genes, environmental factors and genetic and environmental interactions. The unravelling of the genetic background of PCD increasingly contributes to the understanding of observed differences in clinical phenotypes. Although genetic defects in PCD-related genes have robustly been linked to specific ultrastructural anomalies of the ciliary axoneme and a specific ciliary beat pattern, descriptions of the relationship between genotype and clinical phenotype are still scarse [7, 24]. This thesis adds a novel genotype-phenotype relationship to this field. In **chapter 2** we describe the observation that male patients with the *CCDC114* mutation are fertile, in contrast to most other PCD patients. Although this mechanism is not entirely understood yet we provide some evidence for the hypothesis that the function of the *CCDC114* gene may be partially replaced by the *CCDC63* gene, a gene that is 26% identical. This may also explain why the harmful *CCDC114* mutation did not seem to undergo negative selection in the isolated Volendam population. Unfortunately, Knowles and co-workers, which also identified *CCDC114* mutations in a cohort of American PCD patients, did not provide pedigrees that included information on the offspring of patients. Such observations illustrate the importance of gathering comprehensive clinical data to enable comparisons between patients with similar genotypes [25].

1.4 Methodological considerations

The diagnostic approach of PCD has evolved quickly in the past years. Until very recently there was no globally accepted consensus as to which diagnostic results constitute a definite PCD diagnosis, likely diagnosis or an excluded diagnosis [19]. As a result, not all patients have been diagnosed in a similar way and diagnostic delay occurs frequently [26]. Unification of these terms are important in the diagnostic process, the follow-up of patients and enrollment in clinical studies. In **chapter 4** we show that as much as 17% of patients in our cohort was historically diagnosed solely based on clinical symptoms. Although PCD diagnostics in children has been well regulated in the Netherlands for many years, until 2017

it was not recommended by the Dutch Association of Chest Physicians (NVALT) to refer adult patients with non-CF bronchiectasis and clinical suspicion of PCD for diagnostic testing [27]. It is therefore likely that there are many Dutch adult non-CF bronchiectasis patients that are currently unaware of their disease etiology. This may have introduced a bias in our overview of the genetic distribution of Dutch PCD patients in **chapter 4.** In the NVALT guideline, which is currently under revision, it was stated that knowing the underlying etiology of non-CF bronchiectasis would not change the treatment plan as there are no evidence based treatment options. Unfortunately, this kind of self-fulfilling prophecy prevents scientific research to move forward. Shoemark and colleagues showed that identifying the cause of non-CF bronchiectasis in UK patients led to changes in the treatment of ~35% [28]. Further, diagnosing PCD may be important for the general quality of life of patients, for providing the required ENT care and for fertility and genetic counseling [9, 29, 30].

1.5 Recommendations and future perspectives

Genetic testing in PCD is still in its infancy. At this stage, we have only investigated the diagnostic yield of targeted exome sequencing, WES and CNV detection in diagnosed PCD patients, which did not reach beyond 76% [21]. The sensitivity of these techniques in a referral population is still unknown. Therefore, genetic testing cannot yet be a first-line test in PCD diagnostics. However, characterizing the genetic background in all PCD patients is vital to identify the remaining PCD-related genes and thereby improving the diagnostic yield. If genetic testing is used in the diagnostic approach at this point in time, it should be combined with tests that evaluate ciliary structure and function, such as TEM and HVMA [19]. In populations with a high frequency of a specific gene defect, such as in genetic isolates, Sanger sequencing of a single gene or several small genes can be a fast and cost-effective first option (**chapter 2**). In the majority of cases, we recommend WES in combination with an in silico panel of PCD-related genes. This panel can be expanded by a cluster of genes that show increased expression during *in vitro* ciliogenesis, to increase the diagnostic yield of the test (**chapter 4**). Exons of common PCD-related genes, with insufficient reading depth should be analyzed additionally with Sanger sequencing. Further, we recommend to isolate and store RNA from airway cells obtained from ciliary biopsies in all patients as this can be used to study the effect of genomic variants in more detail. In both **chapter 2** and **chapter 3** we show that the effect of splice variants requires confirmation at the RNA level. As in CF, where the sweat test is still needed in some cases, genetic testing is not likely to entirely replace all functional and structural ciliary assessments in PCD in the future. If costs drop and methods for data analysis and storage evolve, whole-genome sequencing may eventually be the preferred test for a genetic diagnosis in all Mendelian diseases, as it gives all the information that our DNA has to offer [31, 32].

Currently, all PCD-related gene defects consist of bi-allelic mutations in one gene or of a mono-allelic mutation in combination with a CNV in the other allele. It is unknown if trans-heterozygous mutations in different PCD-related genes are able to lead to a similar phenotype. In mice, the first evidence was recently provided that trans-heterozygous interactions between *DNAH6* and other PCD genes potentially can cause heterotaxy [33]. Similarly, a subthreshold siRNA knockdown of *Dnah6* in heterozygous *Dnah5* or *Dnai1* mutant mouse respiratory epithelia, causing dual haploinsufficiency, disrupted motile cilia function. There are several reports of PCD patients with only a single heterozygous pathogenic PCD mutation [20–22]. This could reflect the fact that another heterozygous mutation or a CNV has not been found yet or it could indicate that there is a role for transheterozygous mutations. In **chapter 4** we describe that we found single heterozygous mutations in PCD-related genes in 5 patients and did not find any other mutations by additionally sequencing less covered areas of the gene. However, we did not investigate CNVs in these patients. In other ciliopathies involving primary cilia, an oligogenic disease model has been suggested. As an example, in Bardet-Biedl syndrome (BBS) a triallelic inheritance of BBS genes has been proposed as well as a third pathogenic allele that acts as a genetic modifier [34]. Further, in retinitis pigmentosa, transheterozygous mutations in the unlinked RDS and ROM1 gene have been identified, in which generally only compound heterozygotes develop the disease [35] . Therefore, the possibility of an oligogenic disease model in the genetic etiology of PCD should be investigated in the future. This may not only change the way we analyze sequencing data for the diagnosis of PCD but also elucidate novel and more complicated genotype-phenotype relationships explain the observed clinical heterogeneity in PCD.

The ongoing identification of PCD-related genes and mutations has opened up new perspectives for developing personalized genetic therapies, as is seen in CF [36, 37]. Both gene therapy and small molecule cystic fibrosis transmembrane conductance regulator (CFTR) modulators, including potentiators, correctors and translational read-through agents, are designed to treat the underlying cause of CF and have undergone (pre-)clinical testing over the past decade. Ivacaftor, a CFTR potentiator, and Lumacaftor, a CFTR corrector, are currently the only two FDA approved small molecule therapies in CF, increasing chloride transport up to 15-50% of wild type level in patients with class II-IV mutations [37]. Such small molecule compounds that modify the phenotype are generally discovered using high-throughput screens of chemically diverse compounds [38]. Such screens have already been applied in ciliopathy models to identify pathways that are critical for ciliary function [39, 40]. A next step is to investigate whether these drug screens can also be used to select possible targets for therapeutic interventions. As the protein defects in PCD are heterogeneous, development of such therapeutic options should be targeted at the most prevalent gene defects.

Gene therapy can be employed by various techniques that either replace the entire mutated gene, introduce exogenous wildtype mRNA, correct misspliced transcripts or repair the specific gene defect. All these techniques need to be delivered to the cells of interest by a vector. This can either be a viral vector or lipid or polymer nanoparticles. As in CF, the airways are the primary focus of gene therapy in PCD. As the lungs are easily accessible, host defense mechanisms have complicated the use of viral factors while other methods have been slightly more successful [41]. Studies with gene therapy in CF so far have showed limited treatment effects and revealed that there is a need for more efficient vectors to deliver the cDNA to the airway cells [41]. In PCD, four studies investigating gene therapy have been conducted so far [42–45]. Three studies investigated whole-gene replacement of *DNAI1* in mice and cultured human epithelial cells [43–45]. However a number of PCD related genes, of which *DNAH5* is the most important example causing ~30% of cases, exceed the vector capacity as they are too large. Further, replaced and resident cellular genes are driven by different promoters. Subsequently, the expression of a replaced gene may differ and be physiologically unrelated. It also has the risk of incorporating the gene off target, causing a deleterious effect. The most striking example of this risk is the reported vector-induced leukemia through enhancer-mediated mutagenesis in 5 out of 20 children with X-linked severe combined immunodeficiency disorder that received gene therapy [46, 47]. Gene repair does not have these problems as it is not size dependent and does not include integration into the host cell genome. Its feasibility in PCD was demonstrated by correcting *DNAH11* mutations in cultured airway cells from PCD patients normalizing ciliary beat pattern in one third of the cells [42]. A novel gene repair method is provided by CRISPR/CAS9, which was recently discovered as an essential part of adaptive immunity in a bacteria, enabling the organisms to respond to and eliminate invading genetic material. Applying CRISPR/CAS9 to edit mutations in two CF models, a small and large intestinal organoid and induced pluripotent stem cells, demonstrated normal CFTR function [36, 48]. Although the first results are encouraging, this gene repair system also has many challenges to face before it can be safely tested in humans. Moreover, gene repair in an individual with two null mutations can lead to immunological rejection of repaired cells, due to the production of a protein that is not known to the immune system.

PART II RESPIRATORY MANAGEMENT OF PCD

2.1.1 Exhaled breath analysis in PCD

As discussed in more detail in **chapter 5**, metabolites are end products of biochemical processes in our body. Metabolites change in case of disease and are therefore excellent candidates for diagnosing, classifying and following-up disease. The easy access of exhaled

breath (EB) is especially attractive in investigating lung diseases. Specific Volatile Organic Compounds (VOCs) can be identified by using chemical analytical techniques, such as Gas-Chromatography coupled to Mass-Spectrometry or by cross-reactive gas sensors that provide a pattern of sensor responses, electronic noses (eNoses). The potential of exhaled breath analysis has been investigated in the diagnosis of various lung diseases, including acute respiratory stress syndrome, asthma, cancer, chronic obstructive pulmonary disease (COPD) and malignant pleural mesothelioma, on which we have elaborated in **chapter 5**. The distinctive EB patterns in these patients are thought to be a reflection of both the underlying disease processes and the host response. Assessing the breath of children with CF and PCD was a first step in evaluating whether these diseases could also be distinguished from one another by their breath profile (**chapter 6**). Although CF and PCD could be separated on the basis of their breath, we observed a moderate sensitivity of 84% due to some overlap in breath prints. Test characteristics slightly improved when patients with a pulmonary exacerbation were omitted, supporting the notion that some of the discriminating VOCs are related to the host response. The potential benefit of using exhaled breath analysis in CF and PCD primarily lies in non-invasive disease monitoring. Pulmonary exacerbations accelerate disease progression in CF and non-CF bronchiectasis and cause a permanent decline in lung function [49]. Detection of exacerbations during or even prior to arising symptoms would allow clinicians to start adequate therapy timelier. Currently, culture-dependent techniques are used to identify microbes in sputum or throat swabs and direct therapy. This is primarily based on the hypothesis that one primary pathogen drives the immune host response resulting in an increase in sputum, cough and decrease in lung function. However, many studies using novel culture-independent techniques did not observe such changes in the bacterial density or community diversity during exacerbations in CF, compared to a stable disease state [50–52]. These findings suggest that changes in the lung microbial community during exacerbations are far more complex than just the increase of one organism that leads to a systemic inflammatory response. In **chapter 6** we observed distinct breath patterns in children with CF and PCD with clinical signs of a pulmonary exacerbation at the time of the breath collection. As we did not use an analytical molecular technique, we cannot be sure of the exact drivers of these differences. Commonly captured pathogens in CF and PCD, such as *P.aeruginosa* and *S.pneumoniae*, produce several unique VOCs that are not produced in the human body. These VOCs can be accurately detected in the headspace of cultures with different strains [53, 54]. However, available studies comparing *in vitro* data to *in vivo* data observe limited translation into host-pathogen fingerprints. Using a SESI-MS technique, Zhu and colleagues observed that only one quarter to one-third of the total metabolome was shared between the *in vitro* and *in vivo* conditions that were tested [55]. This high degree of variation is likely to be caused by the interaction between bacteria and its environment and by the immune response of the host. It is unlikely that complex

changes that occur during an exacerbation in CF and PCD can be captured by one or several (pre-selected) exhaled VOC biomarkers. In contrast, an unbiased approach, such as provided by eNose technologies that are based on pattern recognition, captures the entire spectrum of contributing VOCs. To investigate the main drivers of a distinct exhaled breath pattern during an exacerbation, these breath patterns should be related to both chemical analytical techniques and microbiome analysis [56]. Such a study is currently underway in children and adults with CF (Merieux study). A next step is to see whether changes can be detected prior to the onset of clinical symptoms. For example, in ventilated ICU patients the slope of the eNose signal can be used to detect patients that develop a ventilator associated pneumonia (VAP) with reasonable accuracy [57]. This may imply that an eNose signal can indeed be used to monitor short-term disease progression in pulmonary disease.

2.1.2 Methodological considerations

Many challenges need to be faced before exhaled breath analysis can reliably be used in a clinical setting (**chapter 5**). Some of which encompass technical issues such as different sampling methods, reproducibility between devices and a lack of standardization in conducting measurements, storage and analysis. Fortunately, important progress is currently made in all these fields by the international breath research community fields. The European Respiratory Society taskforce recently published a set of recommendations on standardization of sample collection and available analytical approaches [58]. These protocols balance between controlling the influence of unwanted noise on the exhaled breath signals and clinical applicability. In **chapter 5** we shortly discuss that efforts have been made by some investigators to control influences, such as background air, exercise, diet and smoking. Although we partially controlled for inhaled substances by letting patients breathe through a filter before exhaling (**chapter 6**), vigorously trying to control all these aspects may hamper clinical usefulness. Moreover, it is still unknown whether fasting or restraining from exercise enhances reproducibility of VOC patterns or improves the signal to noise ratio. When assessing the discriminative ability of exhaled breath analysis in short-term disease progression, such as a pulmonary exacerbation, a first step is to evaluate whether breath profiles are distinct in a cross-sectional study (**chapter 6**). Subsequently, longitudinal analysis in stable disease and during an exacerbation is required to assess normal variability and variability that correlates with clinical progression. As considerable heterogeneity exists in the clinical appearance of pulmonary exacerbations in CF and PCD, the underlying microbial changes and host response, investigations should focus on within-patient changes.

2.1.3 Future perspectives and recommendations

Pattern-recognition based breath tests have the capability to capture the entire spectrum of exhaled metabolites in contrast to analyzing a set of preselected VOCs in breath. However, to investigate what kind of VOCs drive changes in breath prints in different diseases or disease states, it requires combining pattern based techniques with chemical analytical based techniques. Adding other omic -techniques will improve our knowledge on the underlying processes during a pulmonary exacerbation. Further, eNose sensors can be adjusted to increase sensitivity to VOC classes that are the main drivers in a certain breath print, decreasing the signal to noise ratio. Longitudinal data with day to day sampling in CF and PCD patients will shed more light on normal variability and changes that are related to an exacerbation. If technical challenges can be overcome and longitudinal individual changes in a breath print correlate with clinical symptoms, exhaled breath analysis has the potential to development into a point of care test that ideally would facilitate home monitoring of patients.

2.2.1 Hypertonic saline treatment in PCD

As there are no randomised controlled trials in PCD, treatment guidelines are primarily based on extrapolations from CF care and expert opinions [27]. Retained mucus acts as a nidus for chronic infection and subsequent inflammation and lung damage. Muco-active agents may improve sputum cough clearability in patients with increased sputum viscosity, such as in PCD [59–63]. In **chapter 7** we demonstrated that general health perception, as measured by the Quality of Life Bronchiectasis (QoL-B) questionnaire, is modestly improved after 12 weeks of bi-daily inhalations with hypertonic saline in adult PCD patients. However, no change was seen in the primary outcome, the St. George's Respiratory Questionnaire (SGRQ) score and the other secondary outcomes. Being of explorative nature these results need to be interpreted with caution. This study shows a possible benefit of hypertonic saline in PCD patients, but our sample size was small and we tested many variables. The negative result on the primary outcome may be explained by a lack of clinically significant effect of hypertonic saline in PCD patients, but there are also some alternative explanations (**chapter 7**) [64]. One of the main problems that we encountered was the underestimated variability of the outcome parameters, leading to an underpowered study. However, this is the first RCT to include only PCD patients and it provides detailed clinical data that will help the planning of future studies. Further, it raised awareness on the importance of evidence based treatments in PCD. A set of key elements for improving treatment of patients with rare lung diseases were proposed by the PCD research community [64]. One of the recommendations includes the recruitment of pediatric patients as they account for the most diagnosed patients. Another advantage of this would be that initiation of therapy early in the disease may delay further progression. However, despite the dramatic effect that HS has on time to new exacerbation

in CF patients over 6 years of age, no effect was seen in infants with CF in the ISIS trial [65]. These results have spiked the discussion on limitations of current outcome parameters to evaluate early lung disease. A parallel can be drawn to PCD in which lung disease progresses more slowly than in CF and sensitive measures are unavailable at this point in time [66].

2.2.2 Methodological considerations

There is little known about the natural disease course in PCD. Fortunately, recent international collaborations have led to a prospective European patient registration [25]. However, the lack of disease-specific outcome measures in PCD hamper adequate monitoring of disease progression and evaluation of therapeutic options. The ISIS trial illustrated that we need to find measures that are sensitive for changes early in the disease process, when there is no or little permanent lung damage.

Traditional outcome measures of lung health used in clinical trials, such as survival and lung function decline, are too insensitive to be used in short-term trials of therapy for patients with PCD. Spirometry is frequently used in CF and non-CF bronchiectasis patients to monitor disease progression. However, FEV1 values seem relatively insensitive to progression of lung disease in PCD. Evident changes were observed using high-resolution CT (HRCT), while spirometric values remained stable in many patients [67]. Although HRCT is useful for staging, it is impractical for monitoring because of the radiation burden. Lung clearance index (LCI), reflecting ventilation inhomogeneity, correlates much better with HRCT than spirometry in CF patients. It may thus have potential in the follow-up of older children and adults, but measurements are not feasible in infants. In PCD however, the relationship between LCI and structural lung changes is not that clear [68–70]. This underlines the need to standardize equipment and technical methods and to be cautious with extrapolating results from CF research. The US Food and Drug Administration (FDA) released a guidance on patient reported outcomes (PROs), which advocates the use of psychometrically sound PROs in chronic disease conditions [71]. Health-related quality of life (HRQL) measures the impact of disease and treatments on a patients' daily functioning and adds unique information to standard clinical measures. Aztreonam is the first respiratory drug approved by the FDA based on "improvement in respiratory symptoms" as measured by the CFQ-R Respiratory Symptoms Scale [72]. In **chapter 7** we also used a HRQL questionnaire as a primary outcome to explore the effect of hypertonic saline in adult PCD patients. At that time, the St. George's Respiratory Questionnaire (SGRQ) was the best-validated PRO in bronchiectasis patients [73]. It was initially developed for patients with COPD and has a limited number of respiratory symptoms, long and variable recall periods and considerable respondent burden [74]. The Quality of Life Bronchiectasis questionnaire (QoL-B) is developed specifically for non-CF bronchiectasis patients, but was not validated yet at the time the study started [75]. We show some evidence in **chapter 7** that a more disease-specific PRO for PCD, i.e. the QoL-B, was able

to capture significant changes in health perception after hypertonic saline treatment, while the SGRQ was not. This underlines the need for development of sensitive outcome measures in PCD. Recently, the QoL-B PCD has been developed for both children and adults and is now being validated in a large azithromycin trial in PCD [76, 77]. Ideally, a treatment would not only improve daily symptoms but also positively influence long-term disease progression. "Time to new exacerbation" can be regarded as an indirect measure of this process and may qualify as an adequate clinical outcome in PCD [78]. However, exacerbation frequency in PCD is currently unknown, possibly limiting its use [65]. To the best of our knowledge, we are the first to evaluate a range of longitudinal inflammatory markers in PCD (**chapter 7**). Our results confirm the presence of a chronic neutrophilic inflammation in PCD patients [79]. In addition we observed high interferons, possibly linked to an anti-viral response [80].

2.2.3 Recommendations and future perspectives

The mucokinetic effect of HS has been observed *ex vivo* and *in vivo* in healthy controls and various suppurative lung diseases. To study the full potential of HS treatment in PCD, future studies should include a larger sample size. The results described in **chapter 7** of this thesis enable accurate sample size calculations for quality of life, spirometry and inflammatory outcomes. Future studies should also incorporate more disease-specific outcome measures and a longer intervention period.

The study in **chapter 7** raised awareness of the need for improvements in recruitment, development of interventions that account for the multisystem aspects of PCD and sensitive outcome parameters of early lung disease [64]. Multinational studies that include PCD patients that are diagnosed according to recent guidelines are vital to move forward. International patients registration and active participation by patient organizations may help to facilitate trial enrollment to evaluate novel treatment options in PCD [25, 64]. An important example of such a collaboration is the European multicenter RCT on azithromycin maintenance therapy in children and adults with PCD which is currently being conducted [76]. Prophylactic macrolide treatment, covering a wide range of bacteria encountered in PCD, has been studied in many trials in chronic respiratory diseases in the last decade [81]. Improvement in lung function, decrease in pulmonary exacerbations and a reduction in the need for additional antibiotics was observed in CF and non-CF bronchiectasis patients after 6-12 months of use [82–84]. In addition to antibacterial properties there has been increasing interest in the potential anti-inflammatory and immunomodulatory properties of macrolides, possibly attenuating chronic inflammation [81].

FINAL CONCLUSIONS

This thesis provides insight into the genetic background of Dutch PCD patients and the role of a genetic testing in the diagnosis of PCD. The identification of the novel *CCDC114* gene provides a genetic diagnosis in all PCD patients originating from the historically isolated town of Volendam in the Netherlands. In addition, we presented the first X-linked gene that is related to non-syndromic PCD. These findings have a major impact on the analysis of genetic data in PCD and the counseling of patients. At this moment in time, the sole use of a genetic test in the diagnosis of PCD is not feasible as ~20-30% of the PCD-related genes are unknown and technical challenges in the storage, analysis and interpretation of large quantities of sequencing data need to be faced. If these issues can be resolved in the future, whole-exome or whole-genome sequencing in combination with an in-silico gene panel, has the potential to become a first-line diagnostic test in PCD. Until that time, we advise to combine the information obtained from DNA, RNA, functional tests and clinical data to diagnose PCD patients and to improve our knowledge on the genetic base of ciliary function. In this thesis we also show the potential of exhaled breath analysis by eNose in CF and PCD, which primarily lies in non-invasive monitoring of changes in disease status. Longitudinal studies are required to investigate whether this technique can be of any clinical value to the individual patient. If eNose technology is combined with chemical analytical methods to identify the underlying drivers of a breath profile, eNoses can be tailored to detect a certain disease state. This thesis also presents the first RCT in adult PCD patients evaluating the effect of hypertonic saline inhalations on quality of life. Future intervention studies in PCD should include multinational recruitment of patients and use disease-specific outcome measures that are sensitive to early lung disease in PCD and to short-term changes.

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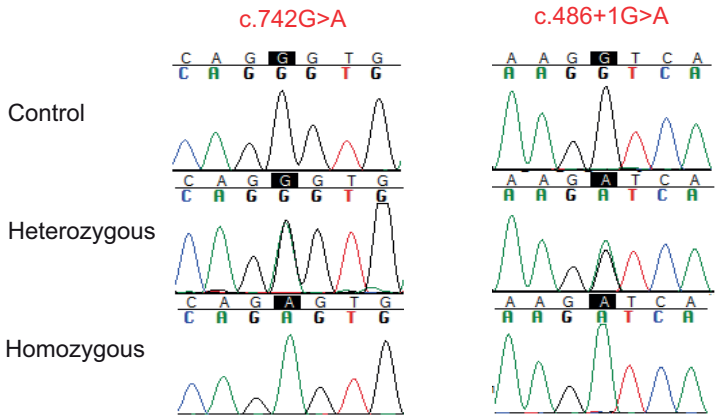
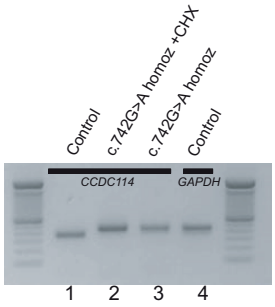
SUPPLEMENTARY FILES



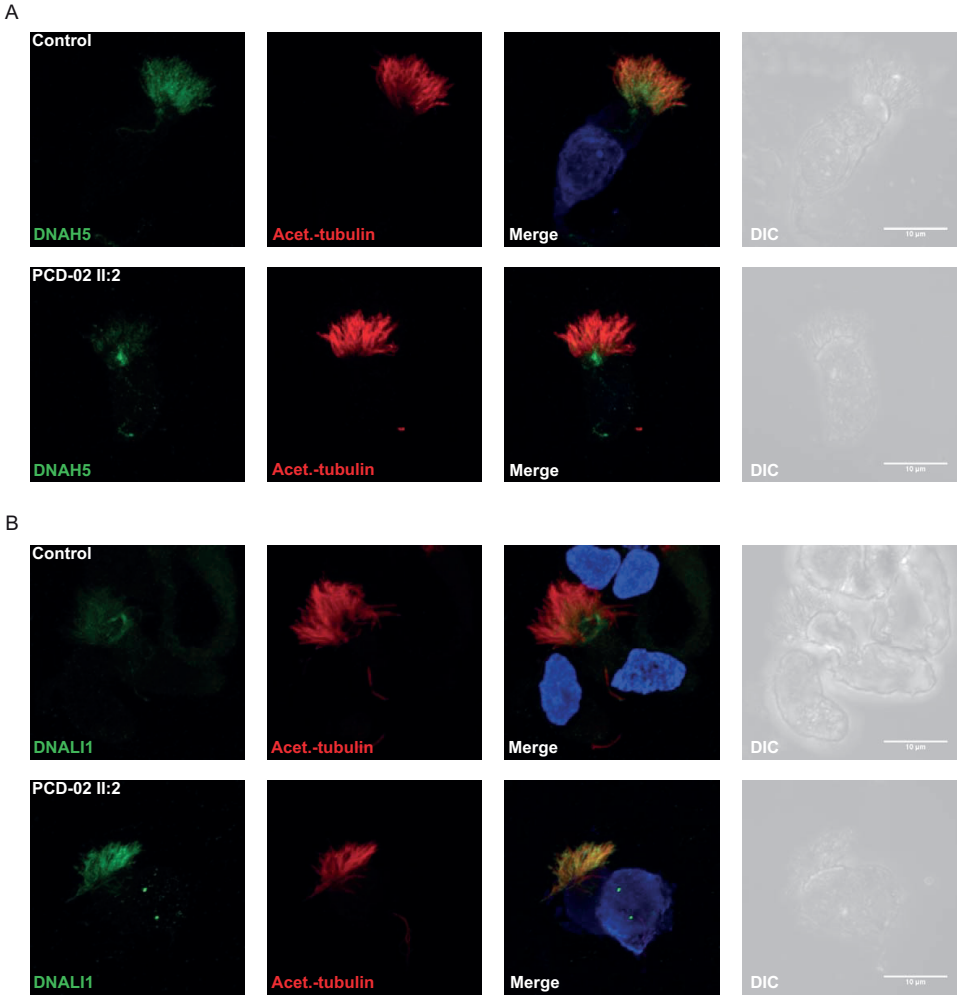
Supplementary figure S2.1. Sequence chromatograms of *CCDC114* mutations

Left panel shows the Volendam mutation and right panel the UK mutation. In both cases a control is shown (upper panels) compared to sequence from a heterozygous (middle panels) and homozygous mutation carrier (lower panels).



Supplementary figure S2.2. RT-PCR showing splice defect arising from Volendam c.742G>A mutation

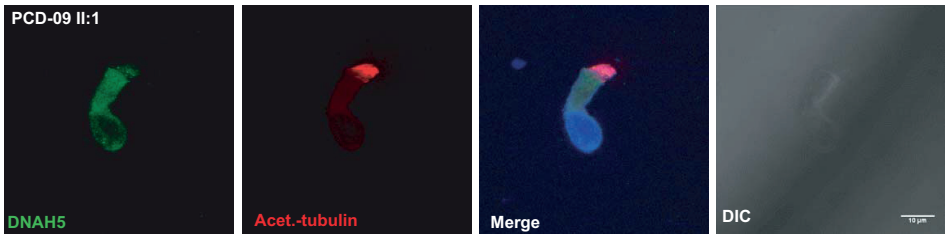
Lanes 1-3, cDNA from control and patient isolated from cultured nasal epithelial cells amplified using primers in *CCDC114* cDNA exon 6 and 8. The control sample was also amplified using *GAPDH* primers as a control (lane 4). A higher molecular weight band is present in the patient cells (lanes 2, 3) indicating an aberrant insertion. Cycloheximide treatment (+CHX) of patient cells (lane 2) to block translation and prevent nonsense-mediated decay of the aberrant mRNA, showed no significant difference in cDNA levels to untreated patient cells (lane 3), indicating no significant effects of nonsense mediated decay; although the untreated band is slightly weaker than CHX-treated. 100 bp. molecular weight marker is shown each side for size reference.



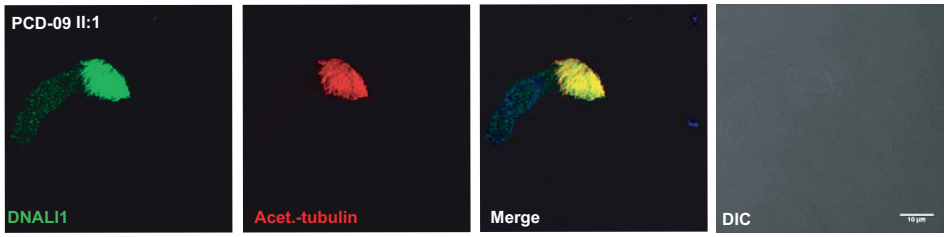
Supplementary figure S2.3. Axonemal immunostaining of ODA and IDA components in Volendam *CCDC114* patient

Subcellular localization of DNAH5 (panel A) and DNALI1 (panel B) in control and patient PCD-02 II:2 respiratory epithelial cells (both in green). Localisation of DNALI1 was not altered in the patient, whereas DNAH5 is markedly reduced compared to the control. Axoneme-specific staining with anti-acetylated α-tubulin antibodies (red) was used as a control, in addition to DNA (DAPI, blue).

A



B



Supplementary figure S2.4. Axonemal immunostaining of ODA and IDA components in UK *CCDC114* patient

Subcellular localization of DNAH5 (panel A) and DNALI1 (panel B) in patient PCD-09 II:1 respiratory epithelial cells (both in green). Localisation of DNALI1 is not altered in the patient, whereas DNAH5 is markedly reduced. Axoneme-specific staining with anti-acetylated α-tubulin antibodies (red) was used as a control, in addition to DNA (DAPI, blue).

Supplementary table S2.1. Exome sequencing coverage and mapping statistics

Exome sequenced samples	CIL5002421	CIL5002422
Read length	76 bp	76 bp
Total bases	6.70 Gb	6.64 Gb
Total reads	88,098,954	87,085,202
Reads mapped	86,774,682 (98.5%)	85,927,959 (98.7%)
%Target bases with coverage >=5X	91.8%	92.3%
%Target bases with coverage >=10X	86.7%	87.4%
%Target bases with coverage >=20X	77.9%	78.9%
Duplication rate	4.39%	5.85%

Supplementary table S2.2. Summary statistics of whole exome filtering process

Filter	Total variants	Variant category
Total variants in CIL5002421	123,880	
Total variants in CIL5002422	123,677	
Total shared variants unfiltered	40,883	
Total shared changes filtered vs. 181 controls	59	
Subset of filtered heterozygous variants	42	
Subset of filtered homozygous variants	11	
Heterozygous variants MAF <0.005	32 (of 42)	
of which		
	1	3′ UTR
	1	5′ UTR
	17	intronic
	4	synonymous
	5	non synonymous
	3	splice site
	1	upstream
Homozygous variants MAF <0.005	4 (of 11)	
of which		
	1	5′ UTR
	2	intronic
	1	non synonymous (*CCDC114*)

Supplementary table S2.3. Primer sequences used for Sanger sequencing of *CCDC114* coding exons

Oligonucleotide	Sequence 5′ to 3′
CCDC114-EX1-F	CCAAAGGGAGCAGAATTCCTA
CCDC114-EX1-R	CAGTCTTCAGCCCCTCTGAC
CCDC114-EX2-F	GTCAGAGGGGCTGAAGACTG
CCDC114-EX2-R	GGCCTAATAGCCCCAATTTC
CCDC114-EX3-F	GCCATTCAATCTCTCCCACA
CCDC114-EX3-R	ATGACCATGCCCAGTTCTTC
CCDC114-EX4-F	GGAACCCCAAAGAACCTCTG
CCDC114-EX4-R	CCTCCATGCCTTTTGGACTA
CCDC114-EX5-F	GCTTTACTCCTTATTGGAGAAGCA
CCDC114-EX5-R	AAGGGGATATTATGGGAGAAAAA
CCDC114-EX6-F	ATCTGGGACACCAGCTGACT

Supplementary table S2.3. Primer sequences used for Sanger sequencing of *CCDC114* coding exons *(Continued)*

Oligonucleotide	Sequence 5' to 3'
CCDC114-EX6-R	AGGGGAAGAGAGAACAGCAG
CCDC114-EX7-F	CCAGGAGGTCTCTGTGTTGG
CCDC114-EX7-R	CAGCCTGCACTGGACTCAG
CCDC114-EX8-F	GCGTCCACTGGCGTCTTA
CCDC114-EX8-R	GGGTGTGGAGCTAGGAAGAA
CCDC114-EX9-F	CCTGCTTCTTCCTAGCTCCA
CCDC114-EX9-R	GTCCTTCCAAGTGGAGAAGC
CCDC114-EX10-11-F	TTGGTCTCTGAGCCTTGACC
CCDC114-EX10-11-R	CCAGCCAGTCCCCAAAAG
CCDC114-EX12-13-F	CCTTTTTGAGGGCTGAGGTC
CCDC114-EX12-13-R	AAAAAGACCCCACAGAGAGC

Supplementary table S2.4. Primer sequences used in RT-PCR

Oligonucleotide	Sequence 5' to 3'
CCDC114_cDNA_2F	AAGATCAGGCGAAGGATCAG
CCDC114_cDNA_5R	CGGTCGTTGTTCTTGAGCTT
CCDC114_cDNA_6F	CTCTCCTCCACCTCTGCCTACGC
CCDC114_cDNA_8R	*A*TGCACCAACAGGTCAGGGTCACT
GAPDH_For	ACCACCAACTGCTTAGCACC
GAPDH_Rev	CACCCTGTTGCTGTAGCCA
ACTB_For	CTGGGACGACATGGAGAAAA
ACTB_Rev	AAGGAAGGCTGGAAGAGTGC

Supplementary table S2.5. Primers and probes in QPCR assays.

Primer	Sequence 5'-3	UPL probe	Genbank
CCDC114-c624F	TACGGGCAAGACTGGAGAGT	#24	NM_144577.3
CCDC114-c672R	CCTTCCATCACTTTGCATTG		
CCDC63-c352F	CAGCAGAAGATTGCGAGTCA	#15	NM_152591.1
CCDC63-c352F	CATGAGACTCAACAGTAGGGTGA		
DNAH5-c5602F	AAAATCATGCAGAAAACTAATCAGG	#1	NM_001369.2
DNAH5-c5648R	TCGTGGTGACGTCTATCAATG		

The Roche UPL (Universal probe library) was used, with the reference gene *ACTB*. The assay was performed on a Lightcycler LC480 (Roche), with assay design via Roche Probefinder vs. 2.48. The PCR cycle was as follows: 10 min 95°C, 1 cycle; 30 sec 60°C, 1 sec 72°C + fluorescence acquisition, 55 cycles. Analysis was performed using Lightcycler software vs. 1.5, with advanced relative quantification mode.

Supplementary table S2.6. UK10K RARE Consortium

Saeed Al-Turki [2,9]	Colin Graham [17]
Carl Anderson [2]	Steve Humphries [7]
Dinu Antony [1]	Matt Hurles [2]
Inês Barroso [2]	Chris Joyce [2]
Phil Beales [1]	Eran Leitersdorf [11]
Jamie Bentham [3]	Shane McCarthy [2]
Stefano Bertolini [14]	Hannah M. Mitchison [1]
Shoumo Bhattacharya [3]	Dawn Muddyman [2]
Sebastiano Calandra [12]	Francesco Muntoni [6]
Keren Carss [2]	Andrew Neil [16]
Krishna Chatterjee [5]	Stephen O'Rahilly [5]
Sebhattin Cirak [6]	Alexandros Onoufriadis [1]
Catherine Cosgrove [3]	Victoria Parker [5]
Allan Daly [2]	Felicity Payne [2]
Petr Danecek [2]	Vincent Plagnol [10]
Richard Durbin [2]	Lucy Raymond [8]
David Fitzpatrick [4]	David B. Savage [5]
Jamie Floyd [2]	Peter Scambler [1]
Reghan Foley [6]	Miriam Schmidts [1]
Chris Franklin [2]	Nadia Schoenmakers [5]
Marta Futema [7]	Mary Seed [13]
Robert Semple [5]	Parthiban Vijayarangakannan [2]
Eva Serra [2]	Klaudia Walter [2]
Jim Stalker [2]	Ros Whittall [7]
Frank Van Bockxmeer [15]	Kathy Williamson [4]
Margriet van Kogelenberg [2]	

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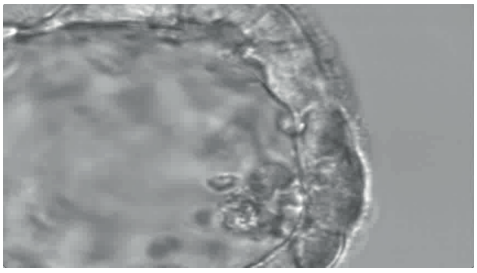
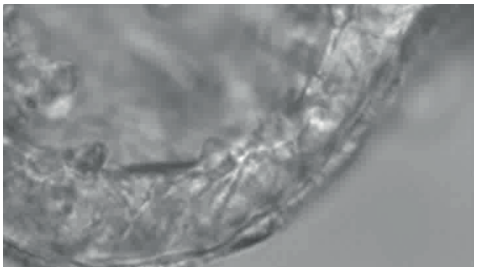
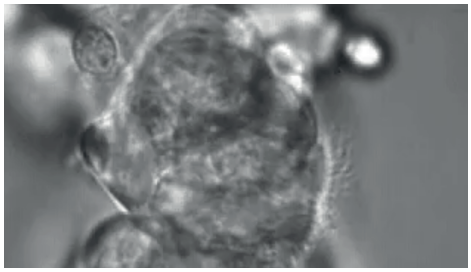


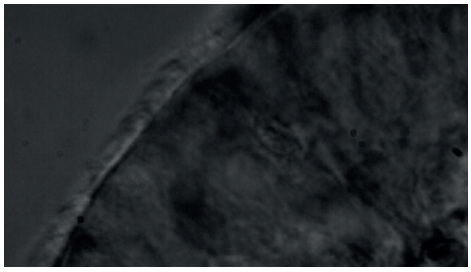
Supplementary movie S2.1. Dutch PCD-01 III:1 nasal cilia



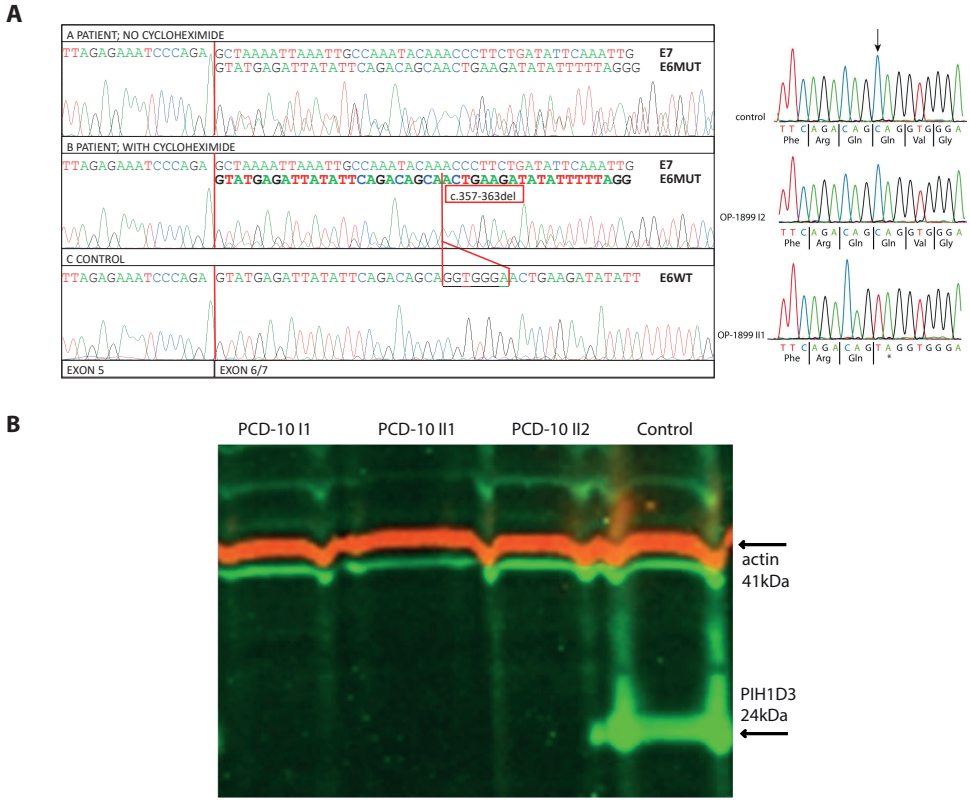
Supplementary movie S2.2. Dutch control nasal cilia



Supplementary movie S2.3. UK PCD-09 II:1 nasal cilia

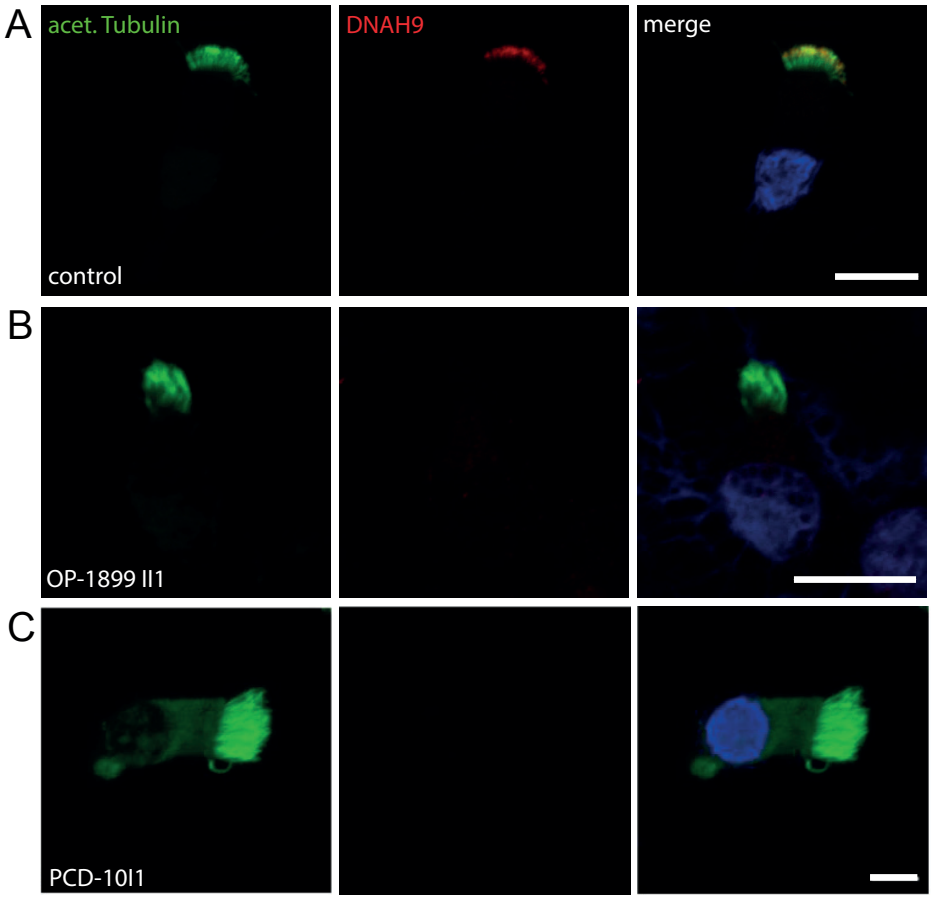


Supplementary movie S2.4. UK control nasal cilia



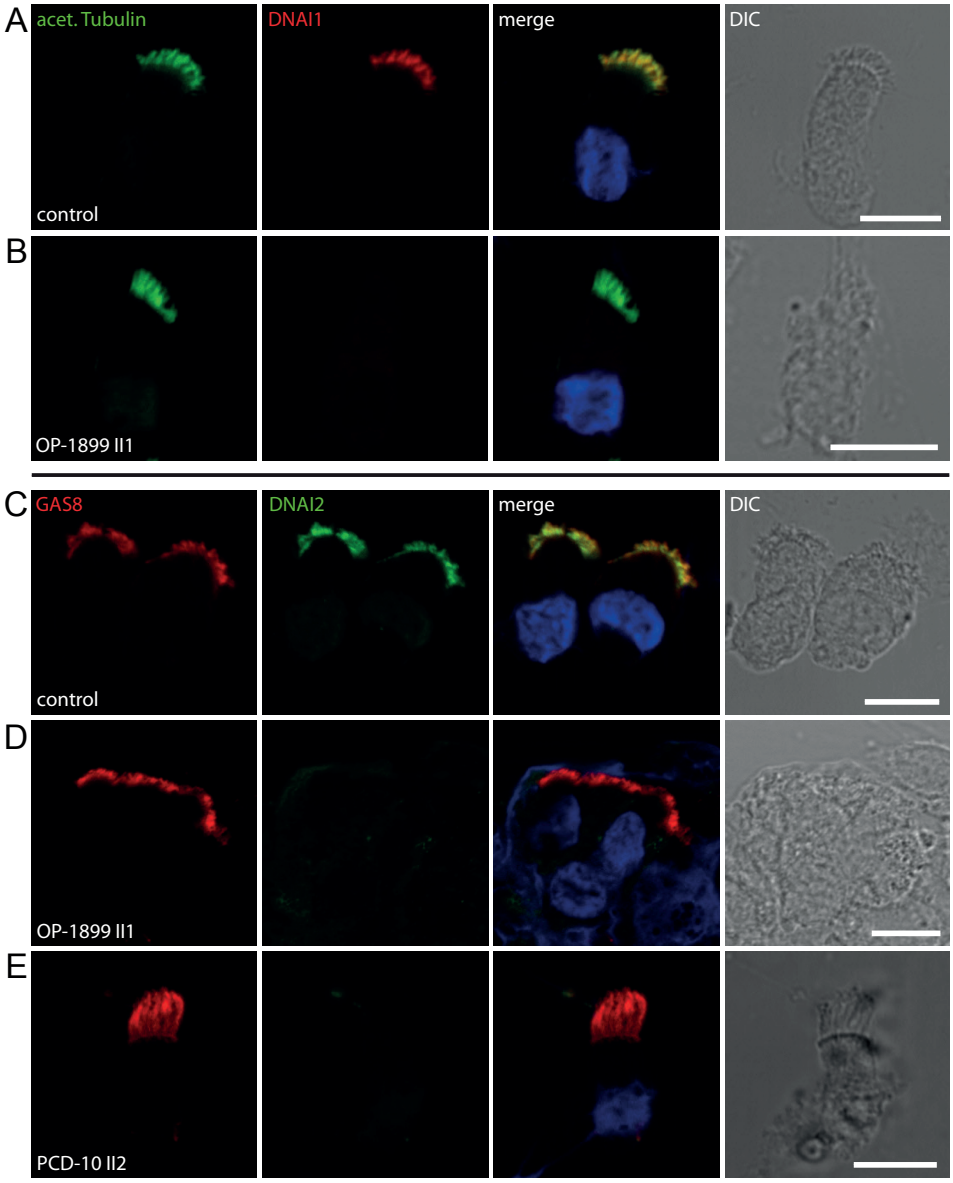
Supplementary figure S3.1. Results of the mutational analyses in PIH1D3 and expression of PIH1D3 in respiratory cells.

(A) cDNA sequences of transcripts from cultured respiratory cells of a patient from family PCD-10, with the c.357_363del variant in PIH1D3 (I), showing two aberrant transcripts: one transcript with the 7 base deletion in exon 6 (E6MUT) and one that shows exon skipping of exon 6 (E7). The cells were cultured with cycloheximide, which blocks the translation step in protein synthesis and consequently inhibits nonsense mediated decay (NMD) (II). This sample shows mainly the E6MUT transcript, indicating that the E6MUT transcript is produced predominantly, but is more subject to NMD in sample A than the exon skipping transcript. In respiratory cells of healthy controls only the wild type (E6WT) transcript is found (III). In family OP-1899 the hemizygous nonsense mutation within exon 6 of PIH1D3 results in a premature stop codon. (B) The expression of PIH1D3 was analyzed by immunoblotting in whole cell lysates of nasal brush biopsies of three patients (PCD-10 I1, PCD-10 II1and PCD-10 II2) with the c.357_363del variant and one healthy control. Actin was used to normalize the amount of protein per well.



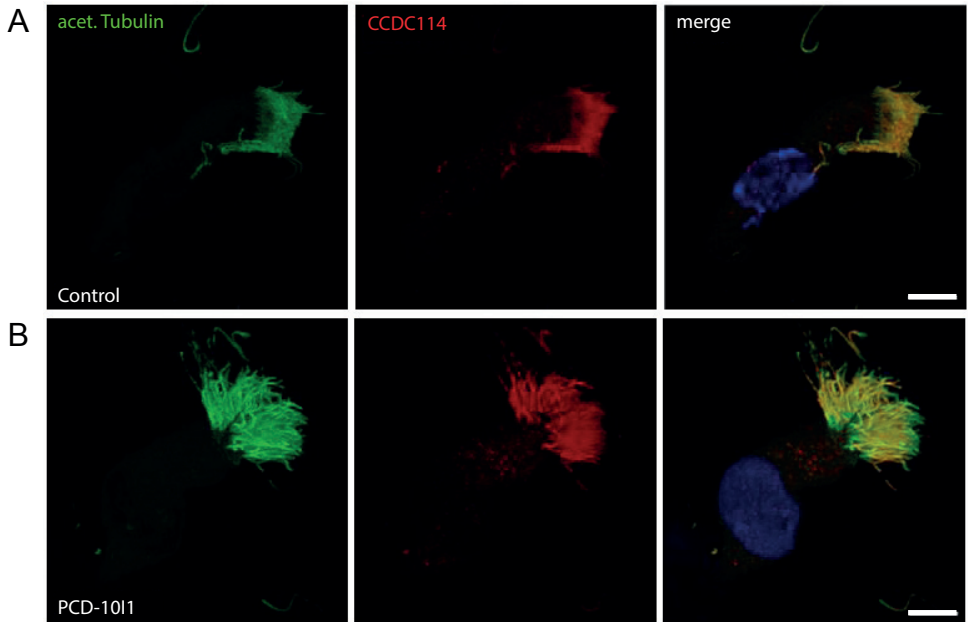
Supplementary figure S3.2. Loss-of-function PIH1D3-mutations result in absence of the outer dynein arm heavy chain DNAH9

(A) Respiratory epithelial cells from control and PCD individuals OP-1899 II1 (B) and PCD-10 I1 (C) were double-labeled with antibodies directed against DNAH9 (red) and acetylated tubulin (green). Acetylated tubulin localizes to the entire length of the cilia, whereas DNAH9 localization is restricted to the distal part of the cilia in control cells (A). In contrast, in PIH1D3-mutant cells, DNAH9 was absent or severely reduced from ciliary axonemes (B-C). Nuclei were stained with Hoechst33342 or DAPI (blue). Scale bars represent 10µm.



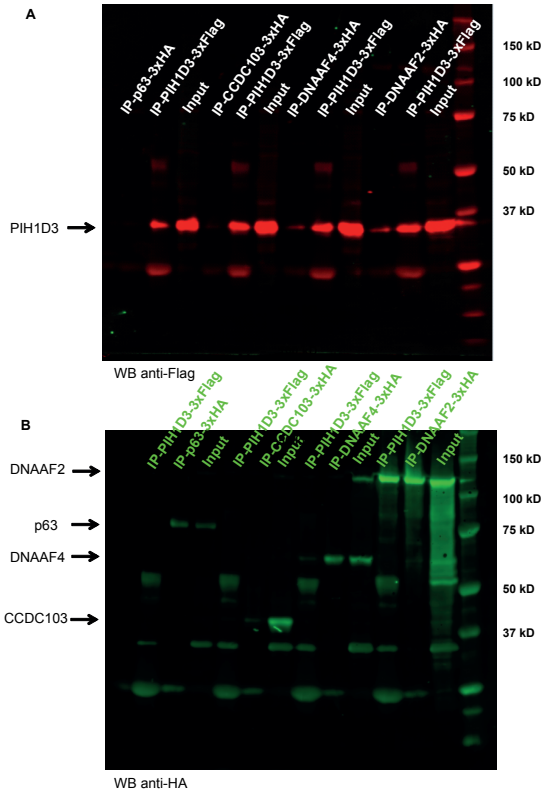
Supplementary figure S3.3. PIH1D3-mutant respiratory cilia are deficient for the outer dynein arm intermediate chains DNAI1 and DNAI2.

Cells were double-labeled with antibodies directed against acetylated tubulin (green) and DNAI1 (red). Both proteins colocalize along the cilia in cells from the unaffected control (yellow) (A). In contrast, in cells of PIH1D3-mutant individuals DNAI1 was absent from or severely reduced in the ciliary axonemes (B). (C-D) Axonemal localization of the ODA intermediate chain DNAI2. Cells were double-labeled with antibodies directed against DNAI2 (green) and GAS8 (red). Both proteins colocalize along the cilia in cells from the unaffected control (yellow) (C). In PIH1D3-mutant respiratory cilia, DNAI2 was absent from or severely reduced in the ciliary axonemes, whereas GAS8 showed a normal distribution pattern (D-E). Nuclei were stained with Hoechst33342 (blue). Scale bars represent 10 µm.



Supplementary figure S3.4. The outer dynein arm docking complex (ODA-DC) is not affected in PIH1D3-mutant cells.

Respiratory cilia were double-labeled with antibodies directed against acetylated tubulin (green) and CCDC114 (red). CCDC114 colocalize with acetylated tubulin along the cilia from unaffected controls (A) and in PIH1D3-mutant axonemes (B) (yellow), indicating that the ODA-DC is not affected by loss of function of PIH1D3. Nuclei were stained with Hoechst33342 (blue). Scale bars represent 10 µm.



Supplementary figure S3.5. Full gel images of the co-immunoprecipitation results shown in Figure 4.

(A) Western-Blot anti-Flag. (B) Western Blot anti HA.

Supplementary table S3.1. Relative mRNA expression of PIH1D3 compared to household gene VCP in c.357_363del mutant cells

	Relative mRNA expression of PIH1D3 (SD)	**Percentage**
With cycloheximide	0.076 (0.005)	100%
Without cycloheximide	0.317 (0.026)	24%

Supplementary table S3.2. Antibodies and dyes

Antibody	**Catalogue number**	**Company**
Rabbit polyclonal anti-PIH1D3	ab151121	Abcam
Mouse monoclonal anti-Actin	ab14128	Abcam
IRDye 800 CW goat anti-rabbit IgG	P/N 925-68071	LI-COR Biosciences
IRDye 800 CW goat anti-rabbit IgG	926-32211	LI-COR Biosciences
IRDye 680 CW goat anti-mouse IgG	P/N 925-68070	LI-COR Biosciences

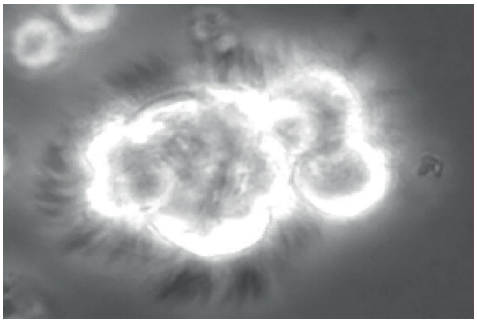
Supplementary table S3.2. Antibodies and dyes *(Continued)*

Antibody	Catalogue number	Company
IRDye 680 CW goat anti-mouse IgG	926-32220	LI-COR Biosciences
Mouse monoclonal anti- acetylated tubulin	T7451	Sigma
Rabbit polyclonal anti-CCDC114	HPA042524	Atlas antibodies
Mouse monoclonal to DNAI2	H00064446-M01	Abnova
Rabbit polyclonal anti-DNAI1	HPA021649	Atlas antibodies
Rabbit polyclonal anti-DNAI2	HPA050565	Atlas antibodies
Rabbit polyclonal anti-DNAH9	HPA052641	Atlas Antibodies
Rabbit polyclonal anti-GAS8	HPA041311	Atlas antibodies
Alexa Fluor 546-conjugated goat antibodies to rabbit	A11035	Life technologies
Alexa Fluor 488-conjugated goat antibodies to mouse	A11029	Life technologies
mouse anti-FLAG	clone M2 F9291)	Sigma
mouse anti-HA	H9658	Sigma
Hoechst33342	14533	Sigma
DAPI	D8417	Sigma Aldrich
Mouse monoclonal anti-DNAH5	[1]	
Rabbit polyclonal anti-DNALI1	[2]	

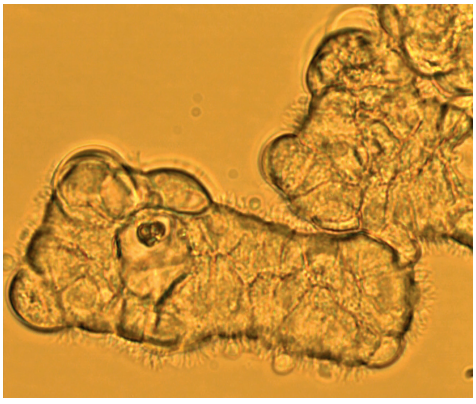
Supplementary table S3.3. Genes tested for interaction with *PIH1D3*

Gene tested	Interaction with ***PIH1D3***
CCDC103	-
CCDC114	-
CCDC151	-
C21orf59	-
DNAAF1	-
DNAAF2	**+**
DNAAF3	-
DNAAF4	**+**
DNAH11	-
DNAI1	-
DNALI1	-
HSP90	**+**
IFT46	-
LRRC6	-
TTC25	-
TXNDC3	-
WDR69	-
ZMYND10	-

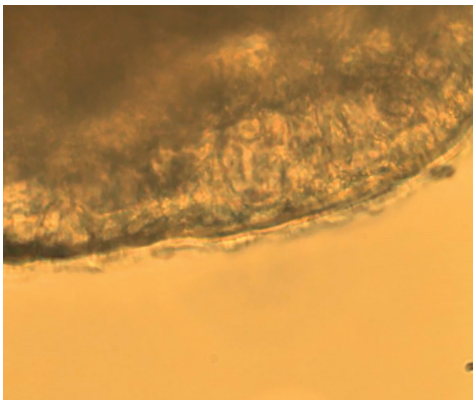
+: interaction observed, -: no interaction observed



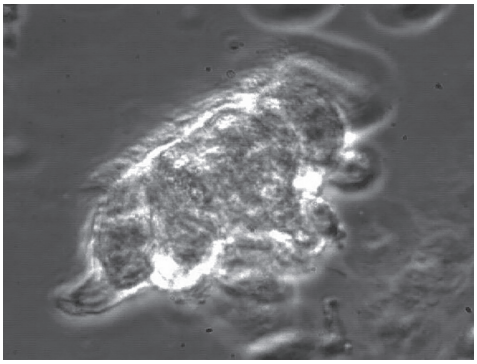
Supplementary movie S3.1. High-speed videomicroscopy of *PIH1D3* mutant respiratory cilia from OP-1899 II1



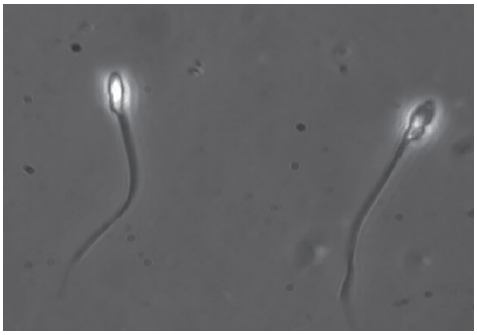
Supplementary movie S3.2. High-speed videomicroscopy of normal respiratory cilia



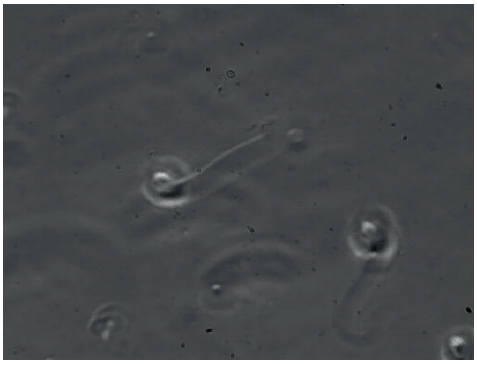
Supplementary movie S3.3. High-speed videomicroscopy of *PIH1D3* mutant respiratory cilia from PCD-10 I1



Supplementary movie S3.4. High-speed videomicroscopy of normal respiratory cilia



Supplementary movie S3.5. High-speed videomicroscopy of *PIH1D3* mutant sperm cell from OP-1899 II1



Supplementary movie S3.6. High-speed videomicroscopy of *PIH1D3* mutant sperm cell from OP-1899 II1

Supplementary table S4.1. Overview of genes included in targeted gene panel

ABHD12B	CATSPERB	DNAH9	IFT140	LRRC77	PPIL6	TEX9
AGR3	CBY1	**DNAI1**	IFT172	LRRC78	PPOX	THNSL1
AK8	**CCDC103**	**DNAI2**	IFT46	LRRC79	PPP1R32	TMEM107
AKAP14	CCDC104	DNAJA1	IFT57	LRRC80	PROM1	TMEM254
ANKMY1	CCDC11	**DNAJB13***	IFT74	LRRC81	RBKS	TMEM67
APOBEC4	**CCDC114**	**DNAL1**	IFT81	LRRC82	RFX3	TNFAIP8L1
ARL3	CCDC135	DNAL4	IFT88	LRRC83	RGS22	TPPP3
ARL6	CCDC146	DNALI1	IL20RA	LRRC84	ROPN1L	TSGA10
ARMC2	CCDC147	DUSP14	INTU	LRRC85	**RPGR**	TSNAXIP1
ARMC4	**CCDC164**	DYDC1	IQCD	LRTOMT	RPGRIP1L	TSPAN6
B9D1	CCDC17	DYNC2H1	IQCG	LRWD1	**RSPH1**	TTC18
B9D2	CCDC170	DYNC2LI1	IQCH	LZTFL1	RSPH10B	TTC21A
BBS10	CCDC176	DYNLL1	IQUB	MAATS1	**RSPH4A**	TTC26
BBS2	CCDC33	DYNLRB1	KATNAL2	MAK	**RSPH9**	TTC29
BBS4	CCDC37	DYNLRB2	KBTBD4	MAP6	RTDR1	TTC30A
BBS5	**CCDC39**	DYRK3	KCNE1	MAPRE3	RUVBL1	TTC30B
BEST4	**CCDC40**	**DYX1C1**	KIF19	MDH1B	RUVBL2	TTC8
BPHL	CCDC41	DZIP1	KIF21A	MEIG1	SLC22A16	TUBA1A
C10orf107	CCDC60	DZIP3	KIF23	MKS1	SLC22A4	TUBB4B
C10orf67	**CCDC65**	EFCAB1	KIF24	MLF1	SLC4A8	TUBD1
C11orf49	CCDC78	EFCAB6	KIF27	MLH1	SMYD2	TUBE1
C11orf63	CCDC81	EFHB	KIF3A	MNS1	SOD1	TUSC3
C11orf65	CCDC89	EFHC1	KIF3B	MORN2	SPA17	UCHL1
C11orf70	CCT6B	EFHC2	KIF6	MORN3	SPAG16	VWA3B
C11orf74	CETN2	ELL3	KIF9	MPDZ	SPAG17	WDPCP
C15orf26	CFTR	ENKD1	KIFAP3	MROH9	SPAG6	WDR16
C1orf158	CLGN	ENKUR	KIFAP4	MSMB	SPAG8	WDR19
C1orf189	CREB3L4	FABP6	KLHDC9	MYCBP	SPATA17	WDR38
C1orf192	CSPP1	FAM154B	KTN1	NEK11	SPATA18	WDR54
C1orf87	CYB5D1	FAM206A	LCA5L	NME5	SPATA4	WDR60
C20orf26	CYB5D2	FAM216B	LRGUK	NME7	SPATA6	WDR78
C20orf85	DAW1	FAM81A	LRRC18	**NME8**	SPATA8	WDR96
C21orf58	DHX40	FBXO15	LRRC23	NPHP1	SPEF1	WRAP53
C21orf59	DIXDC1	FOXJ1	LRRC34	NPHP4	STOML3	WRB
C22orf23	**DNAAF1**	FSIP1	LRRC43	NQO1	STRBP	XRN2
C4orf22	**DNAAF2**	GLB1L	LRRC46	NSUN7	STX2	ZBBX

Supplementary table S4.1. Overview of genes included in targeted gene panel *(Continued)*

C6orf165	**DNAAF3**	GPR162	LRRC48	NUP62CL	TAF1B	ZCWPW1
C9orf116	DNAH10	GPX4	LRRC49	PACRG	TBPL1	**ZMYND10**
C9orf117	**DNAH11**	GSTA1	**LRRC6**	PDE6B	TCTEX1D1	ZMYND12
C9orf135	DNAH12	GSTA3	LRRC71	PFN2	TCTEX1D2	ZNF474
C9orf24	DNAH2	HAGHL	LRRC72	PHTF1	TCTN1	
CALML4	DNAH3	**HEATR2**	LRRC73	PIFO	TCTN1	
CAPS	**DNAH5**	HOOK1	LRRC74	PIH1D2	TEKT1	
CAPSL	DNAH6	**HYDIN**	LRRC75	**PIH1D3***	TEKT2	
CASC1	DNAH7	IFT122	LRRC76	PLEKHB1	TEX26	

Genes in bold are PCD-related genes that were known at the time of creating the targeted gene panel and genes in bold with an asterisk were published as novel PCD-related genes after the creation of the targeted gene panel. CCNO was not included in the initial analysis but was added in a later stage in case of PCD-0030 because of the typical EM and HSVM results.

Supplementary table S4.2. *HYDIN1*-specific gDNA primers

Patient ID	Mutation	Forward primer	Reverse primer
0043	c.8356C>T: p.R2786*	GTAAAACGACGGCCAGTACA-GACCTGGACAACTTCAACGG	CAGGAAACAGCTATGAACCTTGT-TCAAAGTGCCATGGAGG
	c.13867G>T: p.G4622*	GTAAAACGACGGCCAGAGGGAG-GTGAACCTCAGCCT	CAGGAAACAGCTATGAGGTGT-TAACGCTTTAATCATAGAT
1124	c.5388C>A:p.Y1796*	GTAAAACGACGGCCAGAATAGA-TAAGTTTTTTCCCCTCAGCA	CAGGAAACAGCTATGAAGGG-GAAAAAGATGGGAACGTGTAGA
	c.12444-3C>G	GTAAAACGACGGCCAGGTTTTGA-CAAGTTGGCCTA	CAGGAAACAGCTATGAGCTGAGA-TAGATAGCAAGGTC
8078	c.8674_8675delinsG:p.Q2892Gfs*3	GTAAAACGACGGCCAGCTCAT-TCAGTCTTCATACCG	CAGGAAACAGCTATGAAGGAT-GAGTTGGTTACCCTA
8179	c.8674_8675delinsG:p.Q2892Gfs*3	GTAAAACGACGGCCAGCTCAT-TCAGTCTTCATACCG	CAGGAAACAGCTATGAAGGAT-GAGTTGGTTACCCTA
8184	c.8674_8675delinsG:p.Q2892Gfs*3	GTAAAACGACGGCCAGCTCAT-TCAGTCTTCATACCG	CAGGAAACAGCTATGAAGGAT-GAGTTGGTTACCCTA

Supplementary table S4.3. Overview of differential gene expression during in vitro ciliogenesis using RNA sequencing.

This overview shows the logFC and logCPM for both the collagen-monolayer phase (T1) and the suspension phase (T2) for all genes. It shows p value for testing differential expression, the two identified clusters and false discovery rate.

This supplementary .xls file can be found online.

Supplementary methods S7.1.

Participants

Diagnosis was based on a combination of clinical characteristics in combination with at least one of the following; a dysfunction in ciliary motility observed by high-speed videomicroscopy, a ciliary ultrastructural defect observed by electron microscopy or a known genetic defect. Naturally, ciliary motility was not allowed to be normal, but was allowed to be unknown in case a genetic defect or ultrastructural defect was described. Women with a current or intended pregnancy or who were breastfeeding, cigarette smokers and persons with a known quinine sulphate allergy were excluded, as those who had used hypertonic saline, rhDNAse, N-acetylcysteine or non-routine antibiotics in the previous 4 weeks. The forced expiratory volume in 1 s (FEV1) had to be at least 40% of the predicted value for height, age and sex and within 10% of the best value obtained during the previous six months. Participants whose oxygen saturation fell under 90% or whose FEV1 fell more than 15% compared to its prebronchodilator value 15 minutes after inhalation of a test solution with hypertonic saline and taste-masking agent, were not eligible to proceed in the trial.

Randomisation

At the first study visit, all patients were seen by the investigator and checked for eligibility. Upon inclusion participants received a subject identification code coupled to the randomisation list. The randomisation list was generated with 2 equal blocks for 24 eligible patients, accounting for a drop out of 4 patients during the study.

Outcomes and procedures

Each visit consisted of a clinical evaluation, quality of life questionnaires, spirometry, blood sampling, sputum collection following sputum induction and safety checks. Spirometry was performed according to European Respiratory Society standard criteria [3]. Sputum samples for culture and susceptibility testing were collected at baseline and at week 12,16 and 28.

Quality of life questionnaires

Health-related quality of life was measured by two questionnaires. The SGRQ is a self-administered questionnaire designed to measure impact of chest disease on quality of life and has been validated for use in bronchiectasis [4]. It has 50 items with 76 weighted responses divided into 3 categories (symptoms, activity, impact) and requires 8-15 minutes to fill out. The categories are scored separately and can be added to provide a total score ranging from 0 to 100, with 0 indicating no impairment of health-related quality of life. The QoL-B is the first disease specific health related quality of life measure for non-CF bronchiectasis patients [5]. It includes 37 items on 8 scales, Respiratory Symptoms, Physical, Role, Emotional and Social Functioning, Vitality, Health Perception and Treatment Burden. The scores range

from 0-100, with 0 indicating maximum impairment of health related quality of life. Minimal clinically important differences range from 7-10 for the different domains.

Lower respiratory tract infection symptoms

Symptoms were measured using a modified lower respiratory tract infection visual analog scale (LRTI-VAS) which has been previously described [6]. Four of five symptom domains were scored similar to the LRTI-VAS: dyspnea, fatigue, cough, chest pain. Only sputum color was replaced by ease of sputum expectoration, as this was a more valuable item in the context of this study.

Sputum induction

A bronchodilator was used before each sputum induction. Sputum induction was performed by nebulizing a solution of NaCl 3% using the UltraNeb 3000 Ultrasonic Nebuliser (DeVilbiss Healthcare) [7]. After 5 minutes of inhalation participants were instructed to expectorate sputum into a container following huffing exercises. This process was repeated twice. If patients experienced dyspnea, chest tightness or a drop of > 10% was observed in their oxygen saturation, the procedure was paused and spirometry was repeated. If the FEV1 fell by >20%, inhalation was discontinued.

Sputum processing

Sputum samples were processed by the whole-sample technique and liquified using 10 mM dithiothreitol (DTT) as described previously [8]. Supernatants were stored at -80°C till batch-wise analyses. Sputum cell differentiation was performed as described previously [9]. IL-1β, IL-6, IL-8, IL-10, IFN-α, IFN-β and TNF-α were analysed using eBioScience reagents for luminex. Dilutions of samples were performed with 2% (w/v) bovine serum albumin in phosphate-buffered saline, pH 7.2. As DTT treatment of sputum may interfere with antibody-based analyses like luminex, we performed both serial dilutions as well as spike recoveries (recoveries were between 80-120% of expected values). DTT treatment did not bias the detection of the measured mediators, apart from those for IFN-α and IFN-β, the values of which should be regarded as indicative. Enzyme-Linked Immuno Sorbent Assay (ELISA) was used to measure myeloperoxidase (MPO) and neutrophil elastase (NE) as previously described [10].

Pulmonary exacerbations

Pulmonary exacerbation was defined as an acute and significant change in one or more of the common symptoms of bronchiectasis (increase in sputum volume or purulence, worsening dyspnoea, increased cough, declining lung function, increased fatigue/malaise) or the appearance of new symptoms (fever, pleurisy, haemoptysis, requirement for antibiotic

treatment), as described by the British Thoracic Society Guideline for non-CF bronchiectasis [11]. Diary cards with daily report of change in symptoms, unscheduled visits to a clinician and received antibiotics were handed to the investigator at week 12,16,22 and 28. If there was a pulmonary exacerbation patients received care as usual by their attending physician, who was blinded to the patients' treatment allocation. If possible, study medication was continued during an exacerbation.

Statistical analysis

Per-protocol analysis

As this is the first study in PCD patients exploring the effect of hypertonic saline, we performed a per-protocol analysis on data from subjects that completed the entire study and received at least 80% of the study medication, in addition to the intention-to-treat analysis.

Supplementary table S7.1. CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	131
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	132
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	133-134
	2b	Specific objectives or hypotheses	134
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	134-135
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	NA
Participants	4a	Eligibility criteria for participants	133, supplementary files
	4b	Settings and locations where the data were collected	135
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	135
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	135-136, supplementary files
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	136
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	135, supplementary files
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	135, supplementary files

Supplementary table S7.1. CONSORT 2010 checklist of information to include when reporting a randomised trial* *(Continued)*

Section/Topic	Item No	Checklist item	Reported on page No
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	135, supplementary files
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	135
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	135
	11b	If relevant, description of the similarity of interventions	135
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	136
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	136
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	Figure 2
	13b	For each group, losses and exclusions after randomisation, together with reasons	Figure 2, table S7.2
Recruitment	14a	Dates defining the periods of recruitment and follow-up	134-135
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Figure 2
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	137-141, supplementary files
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	NA
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	141

Supplementary table S7.1. CONSORT 2010 checklist of information to include when reporting a randomised trial* *(Continued)*

Section/Topic	Item No	Checklist item	Reported on page No
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	139-141
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	142-144
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	142-144
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	142-144
Other information			
Registration	23	Registration number and name of trial registry	134
Protocol	24	Where the full trial protocol can be accessed, if available	NA
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Supplementary table S7.2. Reasons for declining study participation

Reason to decline participation	Number
Personal reason, unspecified	26
Time spent (study visits / daily study medication)	11
Distance to hospital for study visits	12
Not willing to wash-out/stop hypertonic saline	12
Don't believe it may help	3
Feel too ill to alter current medication	3
Not willing to wash-out/stop acetylcysteine	1
Emigration	1

Supplementary table S7.3. Secondary endpoints

Secondary endpoint Median [IQR]	**Hypertonic (7%) saline**				**Isotonic (0.9%) saline**				**p-value**
	Baseline		**Change 0-12 weeks**		**Baseline**		**Change 0-12 weeks**		
SGRQ (0-100) 100 being worst									
Total	41,8	[32,7-56,2]	-2,6	[-9,0-1,5]	36,4	[28,4-59,1]	-0,3	[-8,2-6,1]	0.33
Activity	59,9	[41,5-70,5]	-5,5	[-17,7-0,2]	55,7	[31,0-70,9]	0,0	[-13,9-5,6]	0.38
Symptoms	56,8	[40,2-74,4]	-2,7	[-11,5-0,7]	59,0	[43,2-72,1]	0,0	[-4,8-5,5]	0.28
Impact	30,1	[18,1-41,9]	-2,2	[-9,7-0,8]	28,1	[18,6-42,8]	-3,3	[-9,6-7,6]	0.25
QoL-B (0-100) 0 being worst									
Role Functioning	66,7	[46,7-86,7]	0,0	[0,0-15,0]	73,3	[66,7-90,4]	-6,7	[-13,3-6,7]	0.05
Vitality	44,4	[27,8-55,6]	11,1	[0,0-22,2]	44,4	[36,1-55,6]	0,0	[-11,1-11,1]	0.15
Emotional Functioning	83,3	[75,0-91,7]	4,1	[-8,3-8,3]	91,7	[75,0-100,0]	-8,3	[-16,7-0,0]	0.16
Health Perception	41,7	[29,2-58,3]	8,3	[0,0-10,4]	50,0	[41,7-58,3]	-8,3	[-16,7-8,3]	0.03*
Physical Functioning	53,3	[26,7-73,3]	0,0	[-6,7-20,0]	63,3	[28,3-88,3]	-6,7	[-20,0-6,7]	0.10
Treatment Burden	55,6	[44,4-77,8]	0,0	[-5,6-11,1]	77,8	[55,6-100]	-11,1	[-33,3-11,1]	0.10
Respiratory Symptoms	48,1	[31,5-64,8]	11,1	[-3,7-25,2]	55,6	[29,6-59,3]	0,0	[-3,7-11,1]	0.36
Social Functioning	75,0	[58,3-83,3]	0,0	[-8,3-8,3]	75,0	[50,0-83,3]	0,0	[-8,3-8,3]	0.68
Modified LTRI-VAS (0-10)									
Dyspnea	4,2	[1,7-5,8]	-0,4	[-2,9-0,6]	3,9	[1,3-5,7]	0,4	[-0,2-1,2]	0.19
Cough	5,0	[3,7-6,8]	-0,8	[-3,4-0,8]	5,3	[3,0-6,5]	0,2	[-1,0-2,3]	0.18
Fatique	5,6	[4,3-7,8]	-1,1	[-3,0-0,7]	4,9	[2,3-5,9]	0,8	[-1,5-1,8]	0.09
Chest pain	1,3	[0,1-3,9]	-0,4	[-1,7-0,2]	0,9	[0,2-2,2]	0,0	[-0,4-0,9]	0.03*
Ease of sputum expectoration	2,1	[1,4-4,7]	0,1	[-2,2-0,9]	2,3	[0,4-4,1]	0,0	[-1,7-2,0]	0.93
Lung function (% pred.)									
FVC	91,0	[83,0-106,0]	-0,5	[-3,0-3,0]	93,5	[82,5-105,0]	-2,0	[-3,0-2,0]	0.36
FEV_1	75,0	[57,5-94,0]	1,5	[-3,5-4,3]	86,0	[55,0-93,8]	0,0	[-3,0-2,0]	0.22
FEF_{25-75}	35,0	[25,3-61,3]	2,0	[-4,5-4,5]	45,0	[26,5-75,3]	1,0	[-8,3-3,5]	0.48

Numerical data are expressed as a median value with interquartile range. * p-value <0.05. Abbreviations: IQR, interquartile range; QoL-B, Quality of Life Bronchiectasis questionnaire; LTRI-VAS, lower respiratory tract infection visual analog scale; % pred., % of predicted value; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; FEF_{25-75}, forced expiratory flow 25-75%.

Supplementary table S7.3. Secondary endpoints *(Continued)*

Secondary endpoint Median [IQR]	Hypertonic (7%) saline				Isotonic (0.9%) saline				p-value
	Baseline		Change 0-12 weeks		Baseline		Change 0-12 weeks		
CRP	0,4	[0,0-6,0]	0,0	[-0,5-1,6]	4,0	[0,0-7,9]	0,0	[-4,0-0,0]	0.16
ESR	8,0	[3,0-11,5]	0,0	[-2,3-4,5]	7,5	[2,0-18,3]	0,0	[-2,0-1,0]	0.29
WBC	8,6	[7,5-11,8]	-0,4	[-1,8-0,5]	8,6	[7,3-10,0]	-0,2	[-1,1-0,7]	0.25
Neutrophils	5,4	[4,4-7,4]	-0,1	[-1,2-0,3]	4,8	[3,9-6,6]	0,1	[-1,1-0,7]	0.30
Eosinophils	0,1	[0,1-0,2]	0,0	[0,0-0,1]	0,1	[0,1-0,2]	0,0	[-0,1-0,0]	0.12
Basophils	0,1	[0,0-0,1]	0,0	[0,0-0,0]	0,1	[0,0-0,1]	0,0	[0,0-0,0]	0.66
Lymphocytes	2,4	[2,1-2,8]	-0,1	[-0,5-0,2]	2,2	[2,0-2,9]	-0,1	[-0,3-0,2]	0.49
Monocytes	0,6	[0,5-0,8]	-0,1	[-0,2-0,1]	0,6	[0,6-0,8]	0,0	[-0,1-0,1]	0.29
Sputum cell differentiation (%)§									
Neutrophils	91,5	[62,5-96,6]	2,9	[0,0-8,3]	92,2	[78,1-97,9]	0,9	[-0,1-4,3]	0.94
Eosinophils	0,4	[0,0-0,5]	-0,1	[-0,3-0,4]	0,2	[0,1-0,6]	0,0	[-0,2-0,3]	0.94
Lymphocytes	0,8	[0,6-1,5]	0,0	[-0,9-0,8]	0,8	[0,4-1,9]	0,2	[-0,4-0,9]	0.43
Macrophages	5,9	[2,3-35,9]	-3,2	[-10,8-0,0]	6,5	[1,3-14,8]	-1,0	[-4,5-0,5]	0.75
Sputum cytokines									
IL-1β (ng/ml)	0,6	[0,01-4,1]	-0,1	[-1,1-0,4]	1,0	[0,2-5,2]	-0,1	[-1,5-0,0]	0.98
IL-6 (ng/ml)	2,8	[0,9-4,7]	-0,5	[-1,3-1,9]	3,8	[2,0-4,9]	-0,5	[-1,3-0,4]	0.06
IL-8 (ng/ml)	13,7	[7,8-34,1]	-3,6	[-7,2-21,7]	24,2	[9,3-40,0]	-6,1	[-18,9-2,1]	0.76
IL-10 (pg/ml)	66,2	[20,3-177,8]	-1,1	[-44,6-86,0]	117,9	[31,2-215,3]	-10,3	[-49,2-2,2]	0.10
TNF-α (ng/ml)	0,3	[0,05-1,4]	0,0	[-0,7-0,3]	0,5	[0,1-1,6]	0,0	[-1,0-0,0]	0.52
NE (ng/ml)	7,9	[1,9-72,7]	-0,4	[-4,1-5,9]	31,4	[2,5-66,4]	-5,7	[-27,4-0,8]	0.10
MPO (ug/ml)	9,2	[2,4-102,1]	-1,3	[-38,8-2,9]	26,2	[3,6-65,7]	-5,1	[-26,8-0,4]	0.90
IFN-α (pg/ml)	38,7	[8,6-91,6]	-4,0	[-23,5-23,5]	68,4	[22,6-107,7]	-11,4	[-18,5-0,6]	0.18
IFN-β (ng/ml)	6,5	[1,6-28,4]	-0,7	[-5,8-6,1]	12,8	[3,3-27,8]	-0,9	[-10,0-0,3]	0.10

Numerical data are expressed as a median value with interquartile range. § basophils, mast cells, plasma cells and monocytes were detected in too small numbers to show. Abbreviations: IQR, interquartile range; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; WBC, white blood cell count; IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; TNF-α, tumor necrosis factor alpha; NE, neutrophil elastase; MPO, myeloperoxidase; IFN-α, interferon alpha; IFN-β, interferon beta.

Supplementary table S7.4. Activities in which participants feel most impaired because of their respiratory symptoms (SGRQ open-ended question)

Activities	N, % of participants
Sports (examples given: cycling, running, softball, swimming, dancing)	19 (86,4%)
Daily activities (examples given: grocery shopping, housekeeping, gardening, climbing stairs, lifting things)	10 (45,5%)
Going out (examples given: going out to dinner, going to a party, being in a smoky room)	12 (54,5%)
Work	6 (27,3%)

Supplementary table S7.5. Observed mean differences, standard deviations and within-patient correlation for the primary and secondary outcomes

Outcome	MCID	Observed mean difference [i.e. (Δ HS) - (Δ control)]	Observed SD	Observed correlation
SGRQ Total	4	-3,5	16,5	0,84
SGRQ_Activity	4	-5,3	21,1	0,83
SGRQ Symtoms	4	-3,7	19,9	0,58
SGRQ_Impact	4	-2,4	19,8	0,83
QoLB Role Functioning	8	12,1	25,3	0,35
QoLB Vitality	10	11,7	38,4	-0,09
QoLB Emotional Functioning	7	3,7	32,0	0,64
QoLB Health Perception	8	10,2	18,9	0,66
QoLB Physical Functioning	10	11,9	32,1	0,88
QoLB Treatment Burden	9	8,3	12,9	0,71
QoLB Respiration Symptoms	8	3,9	28,3	0,64
QoLB Social Functioning	9	-0,6	19,3	0,85
LTRI VAS Dyspnea		-0,9	3,3	0,25
LTRI VAS Cough		-1,3	3,9	0,39
LTRI VAS Fatique		-1,6	3,6	0,57
LTRI VAS Chest pain		-1,9	4,8	0,39
LTRI VAS Ease of sputum exp.		-0,8	5,1	0,01
FVC (% pred.)		1,5	5,6	0,97
FEV_1 (% pred.)		1,2	6,3	0,96
FEF_{25-75} (% pred.)		1,6	13,0	0,95
CRP (mg/liter)		3,5	19,4	0,97
ESR (mm/hour)		2,3	9,8	0,94
WBC (x10e9/L)		-1,1	3,5	0,80
Neutrophils (x10e9/L)		-0,9	3,4	0,78
Eosinophils (x10e9/L)		0,1	0,2	0,17

Supplementary table S7.5. Observed mean differences, standard deviations and within-patient correlation for the primary and secondary outcomes *(Continued)*

Outcome	MCID	Observed mean difference [i.e. (Δ HS) - (Δ control)]	Observed SD	Observed correlation
Basophils (x10e9/L)		0,0	0,0	0,54
Lymphocytes (x10e9/L)		-0,2	0,7	0,37
Monocytes (x10e9/L)		-0,1	0,2	0,47
Sputum Neutrophils (%)		1,8	16,6	0,67
Sputum Eosinophils (%)		0,6	2,1	0,81
Sputum Lymphocytes (%)		-0,3	1,8	0,38
Sputum Macrophages (%)		-2,2	16,4	0,42
Sputum IL-1β ng/ml		0,6	5,1	0,96
Sputum IL6 ng/ml		-0,4	6,3	0,81
Sputum IL-8 ng/ml		50,6	115,1	-0,16
Sputum IL-10 pg/ml		51,1	165,5	0,73
Sputum TNF- α ng/ml		-0,3	2,4	0,90
Sputum NE ng/ml		16,8	31,0	0,34
Sputum MPO µg/ml		1,4	45,9	0,84
Sputum IFN- α pg/ml		10,8	60,3	0,77
Sputum IFN- β ng/ml		9,1	52,2	0,028

Abbreviations: MCID, minimal clinically important difference; HS, hypertonic saline; SD, standard deviation; SGRQ, St. George's Respiratory Questionnaire; QoL-B, Quality of Life Bronchiectasis questionnaire; LTRI-VAS, lower respiratory tract infection visual analog scale; % pred.,% of predicted value; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; FEF_{25-75}, forced expiratory flow 25-75%; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; WBC, white blood cell count; IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; TNF-α, tumor necrosis factor alpha; NE, neutrophil elastase; MPO, myeloperoxidase; IFN-α, interferon alpha; IFN-β, interferon beta.

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ENGLISH SUMMARY

Primary ciliary dyskinesia (PCD) is a rare inherited disease characterized by a defect in motile cilia that line the airways. Cilia sweep the airways by transporting mucus containing inhaled noxious substances, viruses and bacteria. By doing so they are an important part of our innate immune system. PCD patients suffer from mucus that builds up in the airways. As a result they have chronic rhinitis and are vulnerable for infections of the middle ear, sinuses and lungs. Eventually, this leads to permanent lung damage (bronchiectasis) in almost all patients. PCD is difficult to diagnose. In addition, male patients are usually sub –or infertile and in up to 50% of cases, the internal organs are in mirror image. This happens because respiratory cilia, nodal cilia and sperm flagella are remarkably similar in their core structure. Nodal cilia are responsible for the left-right asymmetry of the internal organs.

PCD is difficult to diagnose. The diagnosis is currently made by evaluating ciliary ultrastructure and movement in cells that are obtained by nasal curette biopsy. These tests require expertise, are costly, invasive and the results can take up to several months. A genetic test would be a major improvement in the diagnosis of PCD. However, this is not possible yet as only 60-70% genes that are linked to PCD are known. As motile cilia consist of >200 proteins, many genes can be involved in PCD. After we give an overview of PCD symptoms, diagnostics and treatment options in **chapter 1**, we describe the identification of two novel PCD-related genes in **chapter 2** and **3**. In **chapter 2** we show that a homozygous mutation in the *CCDC114* gene is the cause of PCD in all patients originating from the Dutch town of Volendam. In contrast to an estimated prevalence of 1 in 15,000-30,000 in the rest of the world, 1 in 400 inhabitants of Volendam suffer from PCD. This is related to the settlement of Volendam in 1462. Twenty families founded the village and lived relatively isolated ever since because of geographical, religious and cultural reasons. As a result, several inherited diseases occur more frequently in this population. The identification of the gene defect in *CCDC114* led to the availability of a simple diagnostic genetic test for inhabitants of Volendam. In this chapter we also show that fertility is preserved in PCD patients originating from Volendam, in contrast to fertility of other male PCD patients. The other novel PCD-related gene that we describe in **chapter 3**, *PIH1D3*, lies on the X-chromosome. As a consequence, PCD is inherited in an X-linked recessive manner in patients with this gene defect. This is the first proof of X-linked inheritance in PCD patients without syndromal co-segregation. This finding is important because it influences the analysis of next-generation sequencing data and the counselling of patients on the risks of passing on the disease to their offspring. In **chapter 4** we investigate the diagnostic yield of screening DNA of a Dutch cohort of 74 PCD patients. A preselected part of their DNA was screened by using targeted next-generation sequencing. We tested a panel of 26 genes that were already linked to PCD at that time and 284 PCD candidate genes. We show that in almost 68% of Dutch PCD patients a gene defect can be identified. PCD patients originating from Volendam were not included in this study. PCD candidate genes were selected by investigating differentiating gene expression during *in*

vitro cell culture of nasal curette biopsies from healthy volunteers. Defects in genes with high expression during ciliogenesis could theoretically be related to PCD. The list of approximately 5,500 genes with upregulation during ciliogenesis can now be used in the search for novel PCD-related genes. It may also lead to a reduction of exome sequencing data analysis (in which all coding parts of the DNA is read) to approximately 21% of total

In **chapter 5**, in the second part of this thesis, we summarize the available data on exhaled breath (VOCs; volatile organic compounds) in lung disease. We describe the progress and challenges in capturing and analyzing exhaled breath analysis in the diagnosis and monitoring of pulmonary diseases. In **chapter 6** we show that exhaled breath from children with PCD and CF (cystic fibrosis) can be distinguished from the breath of healthy volunteers and from one another, by use of a so-called "electronic nose". This is an important observation as these diseases can mimic each other in clinic, but have a distinct pathophysiological mechanism. The difference in exhaled breath pattern may reflect this. Moreover, we observed that the breath of patients with a pulmonary exacerbation could be differentiated from the breath of patients in a stable phase. Future research has to evaluate whether individual changes in exhaled breath indicating an exacerbation can be detected. In this way treatment could start earlier. No curative treatment exists for PCD. Due to a lack of scientific evidence, treatments in PCD are extrapolated from CF care. It is important to conduct intervention studies specifically designed for PCD patients. As CF and PCD have a distinct pathophysiology, patients may respond differently to a certain treatment. In **chapter 7** we describe the results of the first randomized controlled trial in adult PCD patients. Inhalations with hypertonic saline, highly concentrated saline water may hydrate airway mucus and thereby lower viscosity. As a result this may improve cough transportability in PCD patients, keeping their airways "cleaner". In 22 PCD patients that received hypertonic and isotonic inhalations (saline water with low concentration) for 12 weeks in a random order, we did not observe a difference in quality of life, based on the "St. George's Respiratory Questionnaire", a questionnaire primarily developed to measure quality of life in COPD patients. The "Quality of Life Bronchiectasis questionnaire", which is more disease-specific but was not yet validated at the time of the study, did show an improvement in general health perception after hypertonic saline treatment. Because we observed large inter-subject variations it is important to use sensitive disease outcome measures and a larger sample size in future PCD studies. In **chapter 8** we reflect on the main results and describe methodological considerations and directions for future research.

The main conclusions derived from the studies of this thesis are:

- One defect in the novel PCD-related gene CCDC114 is the cause of PCD in all PCD patients originating from Volendam. A genetic test has become available for these patients.
- A defect in the novel PCD-related gene PIH1D3 causes recessive X-linked PCD, without syndromal cosegregation. This is in contrast to the (normal) autosomal recessive inheritance mode.
- By using a targeted genepanel including 26 PCD-related genes and 284 candidate genes, we were able to identify the gene defect in almost 68% of a Dutch PCD cohort of 74 patients (who did not originate from Volendam)
- The exhaled breath pattern of VOCs, measured by an electronic nose, was significantly different in children with CF and PCD, compared to healthy children and compared to one another. In addition, exhaled breath patterns of children with and without a pulmonary exacerbation were significantly different in both CF and PCD patients.
- Twelve weeks of hypertonic saline (highly concentrated saline water) nebulizations do not improve quality of life in 22 adult PCD patients, measured by the "St. George's Respiratory Questionnaire". When measured by the Quality of Life Bronchiectasis questionnaire, a more disease-specific questionnaire, we did observe a small positive effect on general health perception after hypertonic saline.

NEDERLANDSE SAMENVATTING

Primaire ciliaire dyskinesie (PCD) is een zeldzame erfelijke aandoening waarbij de trilharen die de luchtwegen bekleden niet goed werken. Trilharen hebben als functie om het slijm in de luchtwegen, waarin ingeademde stoffen, virussen en bacteriën gevangen worden, naar de keel te transporteren. Ze vormen hiermee een belangrijk deel van onze afweer. Bij patiënten met PCD hoopt het slijm in de luchtwegen op. Hierdoor zijn ze vaak chronisch verkouden en veel vatbaarder voor infecties van de oren, bijholtes en longen. Uiteindelijk leidt dit vrijwel altijd tot blijvende longschade (bronchiëctasieën). Tevens zijn mannen vaak verminderd vruchtbaar of onvruchtbaar en zijn in iets minder dan de helft van de gevallen de organen in de borst- en buikholte in spiegelbeeld. Dit wordt veroorzaakt doordat trilharen op het embryo, die verantwoordelijk zijn voor de links-rechts verdeling van de borst -en buikorganen, en de flagella (de zweepstaart die een zaadcel voortbeweegt) een vergelijkbare structuur hebben als de trilharen in de luchtwegen. De diagnose PCD is moeilijk te stellen. Tot nu toe wordt dit gedaan door een schraapsel uit de neus te nemen waarop tests worden uitgevoerd die de beweging en structuur van de trilhaar onderzoeken. Deze tests kunnen alleen door experts gedaan worden, zijn kostbaar, invasief en kosten veel tijd. Een genetische test, die op basis van het uitlezen van het DNA snel kan uitwijzen of iemand PCD heeft, zou een enorme vooruitgang zijn. Dit is echter op dit moment nog niet mogelijk omdat nog maar 60-70% van de ziekte veroorzakende genen bekend zijn. Omdat trilharen uit >200 eiwitten zijn opgebouwd kunnen veel verschillende genen betrokken zijn bij de ziekte. Nadat we in hoofdstuk 1 een overzicht geven van de klinische verschijnselen van PCD, de diagnostiek en behandelmethoden, beschrijven we in hoofdstuk 2 en 3 de ontdekking van twee nieuwe gendefecten die verantwoordelijk zijn voor de ziekte PCD. In **hoofdstuk 2** laten we zien dat een defect in het gen *CCDC114* verantwoordelijk is voor alle ziektegevallen van PCD die in het Nederlandse dorp Volendam voorkomen. In tegenstelling tot in de rest van de wereld, waar PCD bij een geschatte 1 op de 15,000-30,000 mensen voorkomt, komt PCD in Volendam bij 1 op de 400 inwoners voor. Dit heeft te maken met het feit dat Volendam in 1462 door slechts 20 families is gesticht en de bevolking sindsdien relatief geïsoleerd heeft geleefd vanwege geografische, religieuze en culturele redenen. Een aantal erfelijke afwijkingen komt hierdoor vaker voor in Volendam. Door de ontdekking van het gendefect in *CCDC114* kunnen inwoners van Volendam zich nu eenvoudig laten testen op de ziekte PCD. Tevens laten wij in dit hoofdstuk zien dat, in tegenstelling tot de meeste andere mannelijke PCD-patiënten, Volendamse PCD-patiënten vruchtbaar zijn. Het andere gen dat in **hoofdstuk 3** voor het eerst aan PCD gelinkt wordt, *PIH1D3*, is gelegen op het X-chromosoom. Dit zorgt voor een andere overervingsvorm dan tot nu toe beschreven werd bij PCD, een zogenaamde X-gebonden recessieve overerving. Dit is een belangrijke bevinding omdat tot nu toe verondersteld werd dat deze manier van overerving zelden voorkwam bij PCD en alleen in combinatie met een syndromale afwijking. Voor de genetische diagnostiek van patiënten met PCD betekent dit dat de resultaten van het

uitlezen van iemands DNA (middels een techniek die next-generation sequencing heet) op een andere manier geanalyseerd moet worden zodat het gendefect dat verantwoordelijk is voor de ziekte niet over het hoofd gezien wordt. Tevens heeft dit invloed op de manier waarop patiënten gecounseld worden door een erfelijkheidsarts over de risico's op het doorgeven van de ziekte aan kinderen en kleinkinderen. In **hoofdstuk 4** onderzoeken we de diagnostische opbrengst van het screenen van het DNA van een cohort van 74 Nederlandse PCD-patiënten. We hebben van deze patiënten een deel van het DNA uitgelezen middels het gebruik van een zogenaamd next-generation sequencing genpanel van 26 tot dan toe bekende PCD-genen en 284 kandidaat genen. We laten in dit hoofdstuk zien dat het in bijna 68% van de patiënten direct mogelijk is om een ziekte veroorzakend gendefect te identificeren. Patiënten uit Volendam zijn in dit onderzoek niet meegenomen. De kandidaat genen in het genpanel werden geselecteerd door trilhaarcellen van gezonde proefpersonen in het laboratorium te kweken. Er werd vervolgens onderzocht welke genen een verhoogde expressie vertonen tijdens de aanmaak van nieuwe trilharen. Dit zouden namelijk ook de genen kunnen zijn die ervoor kunnen zorgen dat de trilharen niet goed werken, zoals bij PCD. De lijst van in totaal ongeveer 5,500 genen die in dit experiment een verhoogde expressie lieten zien kan gebruikt worden in de zoektocht naar nieuwe PCD-geassocieerde genen. Het kan de grote hoeveelheid aan data, die geproduceerd wordt bij het uitlezen van al het coderende DNA van een persoon (een techniek die exome sequencing heet), reduceren tot ongeveer 21% van totaal. In **hoofdstuk 5**, in het tweede deel van dit proefschrift, wordt het onderzoek op gebied van uitgeademde lucht (VOCs; vluchtige organische metabolieten) bij longziekten samengevat. We beschrijven de voortgang en uitdagingen van het opvangen en analyseren van de uitgeademde lucht voor diagnostiek en monitoring van longziekten. In **hoofdstuk 6** laten wij zien dat kinderen met PCD en CF (cystische fibrose / taaislijmziekte) te onderscheiden zijn van gezonde vrijwilligers en van elkaar op basis van het uitgeademde VOC patroon, gemeten via een "elektronische neus". Dit is een belangrijk gegeven omdat de twee ziektebeelden klinisch op elkaar kunnen lijken, maar een andere oorzaak hebben. Het verschil in het patroon van de uitgeademde vluchtige metabolieten lijkt dit goed te reflecteren. Tevens zagen wij dat de patiënten die tijdens de meting tijdelijk meer last hadden van hun longziekte (die een zogenaamde pulmonale exacerbatie hadden) te onderscheiden waren van de patiënten die dat niet hadden. In de toekomst moet onderzocht worden of een verandering van de uitgeademde lucht bij een patiënt zo'n pulmonale exacerbatie kan voorspellen. Als dit vroegtijdiger opgespoord kan worden dan nu het geval is kan een eventuele behandeling eerder starten.

PCD is tot op heden niet te genezen. Door een gebrek aan wetenschappelijk bewijs worden CF behandelingen ook bij PCD gebruikt. Het is echter belangrijk om studies te verrichten die specifiek voor PCD patiënten ontwikkeld zijn, omdat de twee ziektebeelden een andere oorzaak hebben. Hierdoor kunnen patiënten met PCD anders reageren op behandelingen

dan patiënten met CF. In **hoofdstuk 7** beschrijven we de resultaten van de eerste gerandomiseerde, dubbelblinde behandelstudie bij volwassen PCD patiënten. Vernevelen met hypertoon zout, sterk zoutwater, heeft de potentie om slijm in de luchtwegen minder taai te maken. Hierdoor zouden patiënten met PCD het slijm mogelijk beter op kunnen hoesten en daarmee de luchtwegen "schoner" kunnen houden. Bij 22 PCD-patiënten die in een willekeurige volgorde hypertoon zout en isotoon zout (slap zoutwater) gedurende 12 weken verneveld hebben zagen wij geen duidelijk verschil in kwaliteit van leven op basis van de "*St. George's Respiratory Questionnaire*", een vragenlijst die primair ontwikkeld is om de kwaliteit van leven te meten bij COPD-patiënten. De "*Quality of Life Bronchiectasis questionnaire*", een vragenlijst die waarschijnlijk specifieker is voor het evalueren van klachten die bij PCD vaak voorkomen maar die nog niet gevalideerd was ten tijde van deze studie, liet wel een verbetering van de beleving van de algemene gezondheid zien na vernevelingen met hypertoon zout. De studie laat tevens zien dat PCD-patiënten heel verschillend van elkaar reageren en het daarom belangrijk is om bij een volgend onderzoek goed gevoelige uitkomstmaten te kiezen en een grotere groep patiënten te testen. In **hoofdstuk 8** worden onze studieresultaten in een breder perspectief geplaatst, worden methodologische beperkingen besproken en worden er tevens voorstellen gedaan voor toekomst onderzoek.

De belangrijkste conclusies van dit proefschrift zijn:

- Eén specifiek defect in het nieuw ontdekte PCD-gen *CCDC114* is de oorzaak van PCD bij alle Volendamse PCD-patiënten. Voor deze patiënten is nu een DNA test beschikbaar gekomen.
- Een defect in het nieuw ontdekte PCD-gen *PIH1D3* zorgt voor PCD middels een X-gebonden recessieve overerving, in tegenstelling tot de normale autosomaal recessieve vorm van overerving.
- Middels het uitlezen van DNA met een genpanel van 26 bekende PCD genen en 284 kandidaat genen kan in bijna 68% van een cohort van 74 Nederlandse PCD-patiënten (die niet uit Volendam komen) direct een ziekte veroorzakend gendefect gedetecteerd worden.
- Het patroon van uitgeademde vluchtige organische metabolieten (VOCs), gemeten met een elektronische neus, is verschillend tussen gezonde kinderen, kinderen met CF en kinderen met PCD. Tevens konden we met een elektronische neus onderscheid maken tussen patiënten met CF en PCD die al dan niet een pulmonale exacerbatie hadden.
- Hypertoon zout (sterk zoutwater) vernevelingen gedurende 12 weken geven geen verbetering van kwaliteit van leven ten opzichte van isotoon zout (slap zoutwater) vernevelingen bij een studie met 22 volwassen PCD-patiënten, gemeten met de "St. George's Respiratory Questionnaire". Gemeten met de "Quality of Life Bronchiectasis questionnaire" is er wel een bescheiden positief effect op de beleving van de algemene gezondheid te zien.

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CURRICULUM VITAE

Tamara Paff was born on April 14th, 1986 in Lisse, the Netherlands. In 2004, she graduated *cum laude* from secondary school (Atheneum) at the Katholieke Scholengemeenschap Hoofddorp. In that same year she started medical school at the Leiden University Medical Center. She participated in a pre-clinical elective at the department of Internal Medicine at the Santa Maria University in Lisbon in 2009. During her medical school she developed and supervised a tutor program for first year medical students. In 2010 she was selected for the VVAA/AMREF Flying doctors *Future Leaders Health program* and travelled to rural areas in Tanzania to evaluate sustainability of local projects, together with Dutch and Tanzanian students. That year she did her research internship under the supervision of prof. dr. A.M. Oudesluys-Murphy and prof. dr. N.E. Schalij-Delfos at the department of Paediatrics and Ophthalmology at the Leiden University Medical Center, which sparked her interest in research. Being passionate about paediatrics, she arranged an elective internship in paediatric endocrinology under supervision of prof. dr. M. Dattani at the Great Ormond Street Hospital in Londen in 2010. In 2011, she obtained her medical degree *cum laude* and started her thesis under supervision of prof. dr. P.E. Postmus, dr. E.G. Haarman, dr. J.M.A. Daniels and dr. G. Pals at the VU University Medical Center in Amsterdam. During her PhD project on primary ciliary dyskinesia she obtained a *Fonds NutsOhra grant* (2011), an *International Trainee Scholarship* from the American Thoracic Society (2013), a *best oral presentation award* at the Dutch Longdagen conference (2015) and a *Netherlands Respiratory Society Travel Grant* (2015). From 2014 to 2015 she was a PhD advisory member of the employee's council of the VU University Medical Center. In 2016 she started a residency in paediatrics (ANIOS) at Tergooi in Blaricum (supervisors dr. B.E. van Ewijk / dr. J.J. Jöbsis). Her training in paediatrics started in January 2017 at the Academic Medical Center in Amsterdam (supervisors dr. D.K. Bosman / dr. M.P. Gruppen). During her work as a trainee in paediatrics, she finished her thesis under supervision of prof. dr. Vonk Noordegraaf, dr. E.G. Haarman, dr. J.M.A. Daniels and dr. G. Pals. Tamara married the love of her life, Arend van Deutekom, on December 6th, 2016 on a beach in Ngaio Bay, New Zealand. Tamara Paff-van Deutekom and Arend van Deutekom-Paff live together in Amsterdam.