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GENERAL INTRODUCTION

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Jane S. Lucas; Tamara Paff; Patricia Goggin; Eric G. Haarman.
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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].
CHAPTER 1

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.

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

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, epigenetic 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein class</th>
<th>Ciliary ultrastructural defect by TEM</th>
<th>Ciliary motion defect by HSVM</th>
<th>Clinical phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNAH5</td>
<td>ODA-HC</td>
<td>Absent ODAs</td>
<td>Immotile cilia with occasional stiff cilia</td>
<td>Classic</td>
<td>[70]</td>
</tr>
<tr>
<td>DNAH11</td>
<td>ODA-HC</td>
<td>No defect</td>
<td>Stiff hyperkinetic cilia and cilia with low CBF/immotility</td>
<td>Classic</td>
<td>[72, 75]</td>
</tr>
<tr>
<td>DNAI1</td>
<td>ODA-IC</td>
<td>Absent ODAs</td>
<td>Stiff and immotile cilia</td>
<td>Classic</td>
<td>[71, 76]</td>
</tr>
<tr>
<td>DNAI2</td>
<td>ODA-IC</td>
<td>Absent ODAs</td>
<td>Immotile cilia</td>
<td>Classic</td>
<td>[77]</td>
</tr>
<tr>
<td>NME8</td>
<td>ODA-IC/LC</td>
<td>Absent or shortened ODAs</td>
<td>Normal to immotile cilia</td>
<td>Classic</td>
<td>[78]</td>
</tr>
<tr>
<td>DNAL1</td>
<td>ODA-LC</td>
<td>Absent or shortened ODAs</td>
<td>Low CBF</td>
<td>Classic</td>
<td>[79]</td>
</tr>
<tr>
<td>CCDC39</td>
<td>IDA and N-DRC</td>
<td>Absent IDAs and MTD</td>
<td>Fast, flickery and stiff cilia</td>
<td>Classic</td>
<td>[80]</td>
</tr>
<tr>
<td>CCDC40</td>
<td>IDA and N-DRC</td>
<td>Absent IDAs and MTD</td>
<td>Fast, flickery and stiff cilia</td>
<td>Classic</td>
<td>[81]</td>
</tr>
<tr>
<td>CCDC65</td>
<td>N-DRC</td>
<td>Absent IDAs and MTD</td>
<td>Stiff and dyskinetic cilia</td>
<td>No situs inversus</td>
<td>[82]</td>
</tr>
<tr>
<td>DRC1 (CCDC164)</td>
<td>N-DRC</td>
<td>Absent N-DRC. Single tubuli</td>
<td>Hyperkinetic and stiff cilia</td>
<td>No situs inversus</td>
<td>[83]</td>
</tr>
<tr>
<td>GAS8</td>
<td>N-DRC</td>
<td>Subtle misalignment of outer doublets</td>
<td>Subtle reduction of ciliary amplitude</td>
<td>Classic</td>
<td>[56]</td>
</tr>
<tr>
<td>RSPH1</td>
<td>RS</td>
<td>Ciliary transposition</td>
<td>Low CBF/immotile cilia and stiff cilia with normal CBF</td>
<td>No situs inversus</td>
<td>[54, 55]</td>
</tr>
<tr>
<td>RSPH3</td>
<td>RS</td>
<td>Near absent RS and variable CC defects</td>
<td>Stiff and immotile cilia</td>
<td>No situs inversus</td>
<td>[84]</td>
</tr>
<tr>
<td>RSPH4A</td>
<td>RS</td>
<td>Ciliary transposition</td>
<td>Low CBF/immotile cilia and cilia with circular pattern and normal CBF</td>
<td>No situs inversus</td>
<td>[85]</td>
</tr>
<tr>
<td>RSPH9</td>
<td>RS</td>
<td>Ciliary transposition</td>
<td>Low CBF/immotile cilia and cilia with circular pattern and normal CBF</td>
<td>No situs inversus</td>
<td>[85]</td>
</tr>
</tbody>
</table>
### Table 2. Overview of genes currently linked to primary ciliary dyskinesia. (Continued)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein class</th>
<th>Ciliary ultrastructural defect by TEM</th>
<th>Ciliary motion defect by HSVM</th>
<th>Clinical phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNAJB13</td>
<td>RS/CP</td>
<td>Absent CP</td>
<td>Stiff cilia</td>
<td>No situs inversus</td>
<td>[86]</td>
</tr>
<tr>
<td>HYDIN</td>
<td>CP</td>
<td>CP defect, occasionally ciliary transposition</td>
<td>Stiff cilia with lack of coordination. Occasionally immotile cilia</td>
<td>No situs inversus</td>
<td>[73]</td>
</tr>
<tr>
<td>TTC25</td>
<td>ODA docking protein</td>
<td>Absent ODAs</td>
<td>Immotile cilia or cilia with some residual flickery movement</td>
<td>Classic</td>
<td>[87]</td>
</tr>
<tr>
<td>CCDC103</td>
<td>DA anchoring protein</td>
<td>Absent ODAs + IDAs</td>
<td>Stiff cilia with lack of coordination or immotile cilia</td>
<td>Classic</td>
<td>[88]</td>
</tr>
<tr>
<td>CCDC114</td>
<td>ODA docking protein</td>
<td>Absent ODAs + IDAs</td>
<td>Immotile cilia. Occasionally some twitching cilia</td>
<td>Normal male fertility</td>
<td>[89, 90]</td>
</tr>
<tr>
<td>ARMC4</td>
<td>(Pre-) assembly/transport protein</td>
<td>Absent ODAs</td>
<td>Low CBF and stiff or immotile cilia</td>
<td>Classic</td>
<td>[91]</td>
</tr>
<tr>
<td>C21orf59</td>
<td>(Pre-) assembly protein</td>
<td>Absent ODAs + IDAs</td>
<td>Immotile cilia</td>
<td>Classic</td>
<td>[82]</td>
</tr>
<tr>
<td>CCDC151</td>
<td>(Pre-)assembly protein</td>
<td>Absent ODAs</td>
<td>Immotile cilia</td>
<td>Classic</td>
<td>[92]</td>
</tr>
<tr>
<td>LRRC6</td>
<td>(Pre-) assembly protein</td>
<td>Absent ODAs + IDAs</td>
<td>Immotile cilia</td>
<td>Classic</td>
<td>[93]</td>
</tr>
<tr>
<td>PIH1D3</td>
<td>(Pre-)assembly protein</td>
<td>Absent ODAs + reduced to absent IDAs</td>
<td>Immotile cilia</td>
<td>Classic</td>
<td>[94]</td>
</tr>
<tr>
<td>SPAG1</td>
<td>(Pre-) assembly protein</td>
<td>Absent ODAs + IDAs</td>
<td>Stiff and immotile cilia</td>
<td>Classic</td>
<td>[95]</td>
</tr>
<tr>
<td>ZMYND10</td>
<td>(Pre-) assembly protein</td>
<td>Absent ODAs + IDAs</td>
<td>Low CBF and stiff or immotile cilia</td>
<td>Classic</td>
<td>[96, 97]</td>
</tr>
<tr>
<td>DNAAF1 (LRRC50)</td>
<td>(Pre-) assembly protein</td>
<td>Absent ODAs + IDAs</td>
<td>Immotile cilia</td>
<td>Classic</td>
<td>[98, 99]</td>
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<td>DNAAF2 (KTU)</td>
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<td>Classic</td>
<td>[100]</td>
</tr>
<tr>
<td>DNAAF3</td>
<td>(Pre-) assembly protein</td>
<td>Absent ODAs + IDAs</td>
<td>Immotile cilia</td>
<td>Classic</td>
<td>[101]</td>
</tr>
</tbody>
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<th>Clinical phenotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>DNAAF4 (DYX1C1)</td>
<td>(Pre-) assembly protein</td>
<td>Absent ODAs + IDAs</td>
<td>Low CBF and immotile cilia</td>
<td>Classic</td>
<td>[102]</td>
</tr>
<tr>
<td>DNAAF5 (HEATR2)</td>
<td>(Pre-) assembly protein</td>
<td>Absent ODAs + IDAs</td>
<td>Stiff and immotile cilia</td>
<td>Classic</td>
<td>[103]</td>
</tr>
<tr>
<td>CCNO</td>
<td>Multiciliated cell</td>
<td>RGMC</td>
<td>No defect</td>
<td>No situs inversus</td>
<td>[104]</td>
</tr>
<tr>
<td></td>
<td>differentiation protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCIDAS</td>
<td>Multiciliated cell</td>
<td>RGMC</td>
<td>Immotile cilia</td>
<td>No situs inversus</td>
<td>[105]</td>
</tr>
<tr>
<td></td>
<td>differentiation protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RPGR</td>
<td>NA</td>
<td>Disturbed ciliary orientation</td>
<td>Uncoordinated cilia</td>
<td>Retinitis pigmentosa</td>
<td>[106, 107]</td>
</tr>
<tr>
<td>OFD1</td>
<td>NA</td>
<td>NA</td>
<td>Dyskinetic cilia (no details described)</td>
<td>Macrocephaly, mental retardation</td>
<td>[39]</td>
</tr>
</tbody>
</table>

Abbreviations: 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 underlying 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.
REFERENCES


