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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.

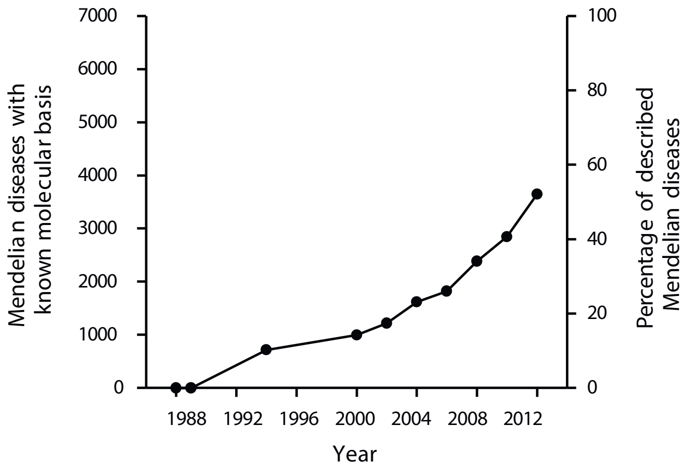

Figure 1. Mendelian diseases of known molecular basis [8].

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.

REFERENCES

1. Kartagener M. Zur Pathogenese der Bronchiektasien: Bronchiectasien bei Situs viscerum inersus. *Beiträge zur Klin. der Tuberkulose* 1933; 83: 489–501.
2. Pennarun G, Escudier E, Chapelin C, Bridoux a M, Cacheux V, Roger G, Clément A, Goossens M, Amselem S, Duriez B. Loss-of-function mutations in a human gene related to Chlamydomonas reinhardtii dynein IC78 result in primary ciliary dyskinesia. *Am. J. Hum. Genet.* 1999; 65: 1508–1519.
3. Loges NT, Olbrich H, Fenske L, Mussaffi H, Horvath J, Fliegauf M, Kuhl H, Baktai G, Peterffy E, Chodhari R, Chung EMK, Rutman A, O'Callaghan C, Blau H, Tiszlavicz L, Voelkel K, Witt M, Zietkiewicz E, Neesen J, Reinhardt R, Mitchison HM, Omran H. DNAI2 mutations cause primary ciliary dyskinesia with defects in the outer dynein arm. *Am. J. Hum. Genet.* 2008; 83: 547–558.
4. Loges NT, Olbrich H, Becker-Heck A, Häffner K, Heer A, Reinhard C, Schmidts M, Kispert A, Zariwala MA, Leigh MW, Knowles MR, Zentgraf H, Seithe H, Nürnberg G, Nürnberg P, Reinhardt R, Omran H. Deletions and point mutations of LRRC50 cause primary ciliary dyskinesia due to dynein arm defects. *Am. J. Hum. Genet.* 2009; 85: 883–889.
5. Duriez B, Duquesnoy P, Escudier E, Bridoux A-M, Escalier D, Rayet I, Marcos E, Vojtek A-M, Bercher J-F, Amselem S. A common variant in combination with a nonsense mutation in a member of the thioredoxin family causes primary ciliary dyskinesia. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104: 3336–3341.
6. Olbrich H, Häffner K, Kispert A, Völkel A, Volz A, Sasmaz G, Reinhardt R, Hennig S, Lehrach H, Konietzko N, Zariwala M, Noone PG, Knowles M, Mitchison HM, Meeks M, Chung EMK, Hildebrandt F, Sudbrak R, Omran H. Mutations in DNAH5 cause primary ciliary dyskinesia and randomization of left-right asymmetry. *Nat. Genet.* 2002; 30: 143–144.
7. Horani A, Ferkol T, Dutcher S, Brody S. Genetics and biology of primary ciliary dyskinesia. *Paediatr. Respir. Rev.* 2016; Mar: 18–24.
8. Brunham LR, Hayden MR. Hunting human disease genes: Lessons from the past, challenges for the future. *Hum. Genet.* 2013; 132: 603–617.
9. Werner C, Onnebrink JG, Omran H. Diagnosis and management of primary ciliary dyskinesia. *Cilia* 2015; 4: 2.
10. Mathijssen IB, Henneman L, van Eeten-Nijman JMC, Lakeman P, Ottenheim CPE, Redeker EJW, Ottenhof W, Meijers-Heijboer H, van Maarle MC. Targeted carrier screening for four recessive disorders: High detection rate within a founder population. *Eur. J. Med. Genet.* Elsevier Masson SAS; 2015; 58: 123–128.
11. Laird NM, Lange C. Family-based designs in the age of large-scale gene-association studies. *Nat. Rev. Genet.* 2006; 7: 385–394.
12. Ferkol TW, Puffenberger EG, Lie H, Helms C, Strauss KA, Bowcock A, Carson JL, Hazucha M, Morton DH, Patel AC, Leigh MW, Knowles MR, Zariwala MA. Primary ciliary dyskinesia-causing mutations in Amish and Mennonite communities. *J. Pediatr.* Elsevier Ltd; 2013; 163: 383–387.
13. Moore A, Escudier E, Roger G, Tamalet A, Pelosse B, Marlin S, Clément A, Geremek M, Delaisi B, Bridoux A-M, Coste A, Witt M, Duriez B, Amselem S. RPGR is mutated in patients with a complex X linked phenotype combining primary ciliary dyskinesia and retinitis pigmentosa. *J. Med. Genet.* 2006; 43: 326–333.

14. Budny B, Chen W, Omran H, Fliegauf M, Tzschach A, Wisniewska M, Jensen LR, Raynaud M, Shoichet SA, Badura M, Lenzner S, Latos-Bielenska A, Ropers H-H. A novel X-linked recessive mental retardation syndrome comprising macrocephaly and ciliary dysfunction is allelic to oral-facial-digital type I syndrome. *Hum. Genet.* 2006; 120: 171–178.
15. Bukowy-Bieryllo Z, Zietkiewicz E, Loges NT, Wittmer M, Geremek M, Olbrich H, Fliegauf M, Voelkel K, Rutkiewicz E, Rutland J, Morgan L, Pogorzelski A, Martin J, Haan E, Berger W, Omran H, Witt M. RPGR mutations might cause reduced orientation of respiratory cilia. *Pediatr. Pulmonol.* 2013; 48: 352–363.
16. Olcese C, Patel MP, Shoemark A, Kiviluoto S, Legendre M, Williams HJ, Vaughan CK, Hayward J, Goldenberg A, Emes RD, Munye MM, Dyer L, Cahill T, Bevillard J, Gehrig C, Guipponi M, Chantot S, Duquesnoy P, Thomas L, Jeanson L, Copin B, Tamalet A, Thauvin-Robinet C, Papon J-F, Garin A, Pin I, Vera G, Aurora P, Fassad MR, Jenkins L, et al. X-linked primary ciliary dyskinesia due to mutations in the cytoplasmic axonemal dynein assembly factor PIH1D3. *Nat. Commun.* 2017; 8: 14279.
17. Olbrich H, Schmidts M, Werner C, Onoufriadis A, Loges NT, Raidt J, Banki NF, Shoemark A, Burgoyne T, Al Turki S, Hurles ME, Köhler G, Schroeder J, Nürnberg G, Nürnberg P, Chung EMK, Reinhardt R, Marthin JK, Nielsen KG, Mitchison HM, Omran H. Recessive HYDIN Mutations Cause Primary Ciliary Dyskinesia without Randomization of Left-Right Body Asymmetry. *Am. J. Hum. Genet.* 2012; 91: 672–684.
18. Pei B, Sisu C, Frankish A, Howald C, Habegger L, Mu XJ, Harte R, Balasubramanian S, Tanzer A, Diekhans M, Reymond A, Hubbard TJ, Harrow J, Gerstein MB. The GENCODE pseudogene resource. *Genome Biol.* 2012; 13: R51.
19. Lucas JS, Barbato A, Collins SA, Goutaki M, Behan L, Caudri D, Dell S, Eber E, Escudier E, Hirst RA, Hogg C, Jorissen M, Latzin P, Legendre M, Leigh MW, Midulla F, Nielsen KG, Omran H, Papon J-F, Pohunek P, Redfern B, Rigau D, Rindlisbacher B, Santamaria F, Shoemark A, Snijders D, Tonia T, Titieni A, Walker WT, Werner C, et al. European Respiratory Society guidelines for the diagnosis of primary ciliary dyskinesia. *Eur. Respir. J.* 2017; 49: pii. 1601090.
20. Djakow J, Kramna L, Dusatkova L, Uhlik J, Pursiheimo JP, Svobodova T, Pohunek P, Cinek O. An effective combination of sanger and next generation sequencing in diagnostics of primary ciliary dyskinesia. *Pediatr. Pulmonol.* 2016; 51: 498–509.
21. Marshall CR, Scherer SW, Zariwala M, Lau L, Paton T, Stockley T, Jobling RK, Ray PN, Knowles MR, Hall D, Dell SD, Kim RH. Whole Exome Sequencing and Targeted Copy Number Analysis in Primary Ciliary Dyskinesia. *G3* 2015; 5: 1775–1781.
22. Boaretto F, Snijders D, Salvoro C, Spalletta A, Mostacciuolo M, Collura M, Cazzato S, Girosi D, Silvestri M, Rossi G, Barbato A, Vazza G. Diagnosis of Primary Ciliary Dyskinesia by a Targeted Next-Generation Sequencing Panel: Molecular and Clinical Findings in Italian Patients. *J. Mol. Diagnostics* 2016; 18: 912–922.
23. Stankiewicz P, Lupski JR. Structural Variation in the Human Genome and its Role in Disease. *Annu. Rev. Med.* 2010; 61: 437–455.
24. Raidt J, Wallmeier J, Hjeij R, Onnebrink JG, Pennekamp P, Loges NT, Olbrich H, Häffner K, Dougherty GW, Omran H, Werner C. Ciliary beat pattern and frequency in genetic variants of primary ciliary dyskinesia. *Eur. Respir. J.* 2014; 44: 1579–1588.
25. Werner C, Lablans M, Ataian M, Raidt J, Wallmeier J, Große-Onnebrink J, Kuehni CE, Haarman EG, Leigh MW, Quittner AL, Lucas JS, Hogg C, Witt M, Priftis KN, Yiallouros P, Nielsen KG, Santamaria F, ÜCkert F, Omran H. An international registry for primary ciliary dyskinesia. *Eur. Respir. J.* 2016; 47: 849–859.

26. Goutaki M, Maurer E, Halbeisen FS, Amirav I, Barbato A, Behan L, Boon M, Casaulta C, Clement A, Crowley S, Haarman E, Hogg C, Karadag B, Koerner-Rettberg C, Leigh MW, Loebinger MR, Mazurek H, Morgan L, Nielsen KG, Omran H, Schwerk N, Scigliano S, Werner C, Yiallouros P, Zivkovic Z, Lucas JS, Kuehni CE. The international primary ciliary dyskinesia cohort (iPCD Cohort): methods and first results. *Eur. Respir. J.* 2017; 49: 1601181.
27. Barbato A, Frischer T, Kuehni C, Snijders D, Azevedo I, Baktai G, Bartoloni L, Eber E, Escribano A, Haarman E, Hesselmar B, Hogg C, Jorissen M, Lucas J, Nielsen K, O'Callaghan C, Omran H, Pohunek P, Strippoli M, Bush A. Primary ciliary dyskinesia: a consensus statement on diagnostic and treatment approaches in children. *Eur. Respir. J.* 2009; 34: 1264–1276.
28. Shoemark A, Ozerovitch L, Wilson R. Aetiology in adult patients with bronchiectasis. *Respir. Med.* 2007; 101: 1163–1170.
29. Pifferi M, Bush A, Di Cicco M, Pradal U, Ragazzo V, Macchia P, Boner L. Health-related quality of life and unmet needs in patients with primary ciliary dyskinesia. *Eur. Respir. J.* 2010; 35: 787–794.
30. Behan L, Dunn Galvin A, Rubbo B, Masefield S, Copeland F, Manion M, Rindlisbacher B, Redfern B, Lucas JS. Diagnosing primary ciliary dyskinesia: an international patient perspective. *Eur. Respir. J.* 2016; 48: 1096–1107.
31. Meienberg J, Bruggmann R, Oexle K, Matyas G. Clinical sequencing: is WGS the better WES? *Hum. Genet.* Springer Berlin Heidelberg; 2016; 135: 359–362.
32. Mallawaarachchi AC, Hort Y, Cowley MJ, McCabe MJ, Minoche A, Dinger ME, Shine J, Furlong TJ. Whole-genome sequencing overcomes pseudogene homology to diagnose autosomal dominant polycystic kidney disease. *Eur. J. Hum. Genet.* 2016; 24: 1584–1590.
33. Li Y, Yagi H, Onuoha EO, Damerla RR, Francis R, Furutani Y, Tariq M, King SM, Hendricks G, Cui C, Saydmohammed M, Lee DM, Zahid M, Sami I, Leatherbury L, Pazour GJ, Ware SM, Nakanishi T, Goldmuntz E, Tsang M, Lo CW. DNAH6 and Its Interactions with PCD Genes in Heterotaxy and Primary Ciliary Dyskinesia. *PLoS Genet.* 2016; 12: 1–20.
34. Katsanis N. The oligogenic properties of Bardet-Biedl syndrome. *Hum. Mol. Genet.* 2004; 13 Spec No: R65-71.
35. Kajiwara K, Berson EL, Dryja TP. Digenic Retinitis Pigmentosa Due to Mutations at the Unlinked Peripherin / RDS and ROM1 Loci. *Science (80-.).* 1994; 264: 1604–1608.
36. Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, Demircan T, Sasaki N, Boymans S, Cuppen E, Van Der Ent CK, Nieuwenhuis EES, Beekman JM, Clevers H. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* Elsevier Inc.; 2013; 13: 653–658.
37. Quon BS, Rowe SM. New and emerging targeted therapies for cystic fibrosis. *BMJ* 2016; 352: i859.
38. Van Goor F, Straley K, Cao D, González J, Hadida S, Hazlewood A, Joubran J, Knapp T, Makings L, Miller M, Neuberger T, Olson E, Panchenko V, Rader J, Singh A, Stack J, Tung R, Grootenhuis P, Negulescu P. Rescue of DF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2006; 290: L1117-1130.
39. Avasthi P, Marshall WF. Chemical screening methods for flagellar phenotypes in chlamydomonas. Methods Enzymol. 2013.
40. Avasthi P, Marley A, Lin H, Gregori-puigjane E, Brian K, Zastrow M Von, Marshall WF. A chemical screen identifies class A G-protein coupled receptors as regulators of cilia. *ACS Chem. Biol.* 2013; 7: 911–919.

41. Alton EWFW, Boyd AC, Davies JC, Gill DR, Griesenbach U, Harrison PT, Henig N, Higgins T, Hyde SC, Innes JA, Korman MSD. Genetic medicines for CF: Hype versus reality. *Pediatr. Pulmonol.* 2016; 51: S5–S17.
42. Lai M, Pifferi M, Bush A, Piras M, Michelucci A, Di Cicco M, Del Grosso A, Quaranta P, Cursi C, Tantillo E, Franceschi S, Mazzanti MC, Simi P, Saggese G, Boner A, Pistello M. Gene editing of DNAH11 restores normal cilia motility in primary ciliary dyskinesia. *J Med Genet* 2016; 53: 242–249.
43. McIntyre JC, Davis EE, Joiner A, Williams CL, Tsai I-C, Jenkins PM, McEwen DP, Zhang L, Escobado J, Thomas S, Szymanska K, Johnson CA, Beales PL, Green ED, Mullikin JC, Sabo A, Muzny DM, Gibbs RA, Attié-Bitach T, Yoder BK, Reed RR, Katsanis N, Martens JR. Gene therapy rescues cilia defects and restores olfactory function in a mammalian ciliopathy model. *Nat. Med.* 2012; 18: 1423–1428.
44. Chhin B, Negre D, Merrot O, Pham J, Tourneur Y, Ressnikoff D, Jaspers M, Jorissen M, Cosset F-L, Bouvagnet P. Ciliary beating recovery in deficient human airway epithelial cells after lentivirus ex vivo gene therapy. *PLoS Genet.* 2009; 5: e1000422.
45. Ostrowski L, Yin W, Patel M, Sechelski J, Rogers T. Restoring ciliary function to differentiated Primary Ciliary Dyskinesia cells with a lentivector. *Gene Ther.* 2014; 21: 253–261.
46. Howe SJ, Mansour MR, Schwarzwaelder K, Hubank M, Kempski H, Brugman MH, Ridder D De, Gilmour KC, Adams S, Thornhill SI, Parsley KL, Staal FJT, Rosemary E, Linch DC, Bayford J, Brown L, Quaye M, Kinnon C, Ancliff P, Webb DK, Schmidt M, Kalle V, Gaspar HB, Thrasher AJ. Insertional mutagenesis in combination with acquired somatic mutations leads to leukemogenesis following gene therapy of SCID-X1. *J. Clin* 2008; 118: 3143–3150.
47. Hacein-bey-abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, Clappier E, Caccavelli L, Delabesse E, Beldjord K, Asnafi V, Macintyre E, Cortivo LD, Radford I, Brousse N, Wintergerst U, Velez MC, Leiva L, Sorensen R, Wulffraat N. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J. Clin. Invest.* 2008; 118: 3132–3142.
48. Firth AL, Menon T, Parker GS, Qualls SJ, Lewis BM, Ke E, Dargitz CT, Wright R, Khanna A, Gage FH, Jolla L, Clinic C. Functional Gene Correction for Cystic Fibrosis in Lung Epithelial Cells Generated From Patient iPSCs. *Cell Rep.* 2015; 12: 1385–1390.
49. Waters V, Stanojevic S, Atenafu EG, Lu A, Yau Y, Tullis E, Ratjen F. Effect of pulmonary exacerbations on long-term lung function decline in cystic fibrosis. *Eur. Respir. J.* 2012; 40: 61–66.
50. Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, Cavalcoli JD, VanDevanter DR, Murray S, Li JZ, Young VB, LiPuma JJ. Decade-long bacterial community dynamics in cystic fibrosis airways. *Proc. Natl. Acad. Sci. U. S. A.* 2012; 109: 5809–5814.
51. Stressmann FA, Rogers GB, Marsh P, Lilley AK, Daniels TW V, Carroll MP, Hoffman LR, Jones G, Allen CE, Patel N, Forbes B, Tuck A, Bruce KD. Does bacterial density in cystic fibrosis sputum increase prior to pulmonary exacerbation? *J. Cyst. Fibros.* 2011; 10: 357–365.
52. Price KE, Hampton TH, Gifford AH, Dolben EL, Hogan DA, Morrison HG, Sogin ML, O'Toole GA. Unique microbial communities persist in individual cystic fibrosis patients throughout a clinical exacerbation. *Microbiome* 2013; 1: 27.
53. Zhu J, Bean HD, Kuo Y-M, Hill JE. Fast detection of volatile organic compounds from bacterial cultures by secondary electrospray ionization-mass spectrometry. *J. Clin. Microbiol.* 2010; 48: 4426–4431.
54. Bos LDJ, Sterk PJ, Schultz MJ. Volatile metabolites of pathogens: a systematic review. *PLoS Pathog.* 2013; 9: e1003311.
55. Zhu J, Bean HD, Wargo MJ, Leclair LW, Hill JE. Detecting bacterial lung infections: in vivo evaluation of in vitro volatile fingerprints. *J. Breath Res.* 2013; 7: 16003.

56. Shaw DE, Sousa AR, Fowler SJ, Fleming LJ, Roberts G, Corfield J, Pandis I, Bansal AT, Bel EH, Auffray C, Compton CH, Bisgaard H, Bucchioni E, Caruso M, Chanez P, Dahlén B, Dahlen SE, Dyson K, Frey U, Geiser T, De Verdier MG, Gibeon D, Guo YK, Hashimoto S, Hedlin G, Jeyasingham E, Hekking PPW, Higenbottam T, Horváth I, Knox AJ, et al. Clinical and inflammatory characteristics of the European U-BIOPRED adult severe asthma cohort. *Eur. Respir. J.* 2015; 46: 1308–1321.
57. Bos L, Martin-Loeches I, Kastelijn J, Gili G, Espasa M, Povoa P, Kolk A, Janssen H, Sterk P, Artigas A, Schultz M. The volatile metabolomic fingerprint of ventilator-associated pneumonia. .
58. Horváth I, Barnes, Peter J, Loukides S, Sterk PJ. A European Respiratory Society technical standard: exhaled biomarkers in lung disease. *Eur. Respir. J.* 2017; 49: pii: 1600965.
59. Kellett F, Redfern J, Niven R. Evaluation of nebulised hypertonic saline (7%) as an adjunct to physiotherapy in patients with bronchiectasis. *Respir. Res.* 2005; 99: 27–31.
60. King M, Dasgupta B, Tomkiewicz RP, Brown NE. Rheology of cystic fibrosis sputum after in vitro treatment with hypertonic saline alone and in combination with recombinant human deoxyribonuclease I. *Am. J. Respir. Crit. Care Med.* 1997; 156: 173–1777.
61. Robinson M, Hemming AL, Regnis JA, Wong a G, Bailey DL, Bautovich GJ, King M, Bye PT. Effect of increasing doses of hypertonic saline on mucociliary clearance in patients with cystic fibrosis. *Thorax* 1997; 52: 900–903.
62. Daviskas E, Anderson SD. Hyperosmolar agents and clearance of mucus in the diseased airway. *J. aerosol Med.* 2006; 19: 100–109.
63. Bilton D, Tino G, Barker AF, Chambers DC, De Soyza A, Dupont LJA, O'Dochartaigh C, van Haren EHJ, Vidal LO, Welte T, Fox HG, Wu J, Charlton B. Inhaled mannitol for non-cystic fibrosis bronchiectasis: a randomised, controlled trial. *Thorax* 2014; 69: 1073–1079.
64. Kuehni CE, Goutaki M, Kobbernagel HE. Hypertonic saline in patients with primary ciliary dyskinesia: on the road to evidence-based treatment for a rare lung disease. *Eur. Respir. J.* 2017; 49.
65. Rosenfeld M, Ratjen F, Brumback L, Daniel S, Rowbotham R, McNamara S, Johnson R, Kronmal R, Davis S, ISIS Study Group. Inhaled hypertonic saline in infants and children less than six years of age with cystic fibrosis: the ISIS randomized trial. *J. Am. Med. Assoc.* 2012; 307: 2269–2277.
66. Sagel SD, Davis SD, Campisi P, Dell SD. Update of respiratory tract disease in children with primary ciliary dyskinesia. *Proc. Am. Thorac. Soc.* 2011; 8: 438–443.
67. Maglione M, Bush A, Montella S, Mollica C, Manna A, Esposito A, Santamaria F. Progression of lung disease in primary ciliary dyskinesia: Is spirometry less accurate than CT? *Pediatr. Pulmonol.* 2011; : 1–7.
68. Irving SJ, Ives A, Davies G, Donovan J, Edey AJ, Gill SS, Nair A, Saunders C, Wijesekera NT, Alton EWFW, Hansell D, Hogg C, Davies JC, Bush A. Lung Clearance Index and High-Resolution Computed Tomography Scores in Primary Ciliary Dyskinesia. *Am. J. Respir. Crit. Care Med.* 2013; 188: 545–549.
69. Green K, Buchvald FF, Marthin JK, Hanel B, Gustafsson PM, Nielsen KG. Ventilation inhomogeneity in children with primary ciliary dyskinesia. *Thorax* 2012; 67: 49–53.
70. Boon M, Vermeulen FL, Gysemans W, Proesmans M, Jorissen M, De Boeck K. Lung structure-function correlation in patients with primary ciliary dyskinesia. *Thorax* 2015; 70: 339–345.
71. U.S. Department of Health and Human Services; Food and Drug Administration. Patient-reported outcome measures: use in medical product development to support labeling claims.
72. Retsch-Bogart GZ, Quittner AL, Gibson RL, Oermann CM, McCoy KS, Montgomery AB, Cooper PJ. Efficacy and safety of inhaled aztreonam lysine for airway pseudomonas in cystic fibrosis. *Chest* 2009; 135: 1223–1232.

73. Wilson CB, Jones PW, Leary CJO, Cole PJ, Wilson R. Validation of the St. George ' s Respiratory Questionnaire in Bronchiectasis. *Am. J. Respir. Crit. Care Med.* 1997; 156: 536–541.
74. Jones PW. St. George's Respiratory Questionnaire: MCID. *COPD J. Chronic Obstr. Pulm. Dis.* 2005; 2: 75–79.
75. Quittner AL, O'Donnell AE, Salathe M, Lewis S, Li X, Montgomery B, O'Riordan TG, Barker AF. Quality of Life Questionnaire-Bronchiectasis: final psychometric analyses and determination of minimal important difference scores. *Thorax* 2015; 70: 12–20.
76. Kobbernagel HE, Buchvald FF, Haarman EG, Casaulta C, Collins S a., Hogg C, Kuehni CE, Lucas JS, Omran H, Quittner AL, Werner C, Nielsen KG. Study protocol, rationale and recruitment in a European multi-centre randomized controlled trial to determine the efficacy and safety of azithromycin maintenance therapy for 6 months in primary ciliary dyskinesia. *BMC Pulm. Med.* BMC Pulmonary Medicine; 2016; 16: 104.
77. Lucas JS, Behan L, Galvin AD, Alpern A, Morris AM, Carroll MP, Knowles MR, Leigh MW, Quittner AL. A quality-of-life measure for adults with primary ciliary dyskinesia: QOL – PCD. *Eur. Respir. J.* 2015; 46: 375–383.
78. Elkins M, Robinson M, Rose B, Harbour C, Moriarty C, Marks G, Belousova E, Xuan W, Bye P, National Hypertonic Saline in Cystic Fibrosis Study Group. A controlled trial of long-term hypertonic saline in patients with cystic fibrosis. *New Engl. Med. J.* 2006; 354: 229–240.
79. Ratjen F, Waters V, Klingel M, McDonald N, Dell S, Leahy T, Yau Y, Grasemann H. Changes in airway inflammation during pulmonary exacerbations in patients with cystic fibrosis and primary ciliary dyskinesia. *Eur. Respir. J.* 2016; 47: 829–836.
80. Gao YH, Guan WJ, Xu G, Lin ZY, Tang Y, Lin ZM, Gao Y, Li HM, Zhong NS, Zhang GJ, Chen RC. The role of viral infection in pulmonary exacerbations of bronchiectasis in adults: A prospective study. *Chest* 2015; 147: 1635–1643.
81. Spagnolo P, Fabbri LM, Bush A. Long-term macrolide treatment for chronic respiratory disease. *Eur. Respir. J.* 2013; 42: 239–251.
82. Southern K, Barker P, Solis-Moya A, Patel L. Macrolide antibiotics for cystic fibrosis. *Cochrane database Syst. Rev.* 2011; 11: CD002203.
83. Wong C, Jayaram L, Karalus N, Eaton T, Tong C, Hockey H, Milne D, Fergusson W, Tuffery C, Sexton P, Storey L, Ashton T. Azithromycin for prevention of exacerbations in non-cystic fibrosis bronchiectasis (EMBRACE): a randomised, double-blind, placebo-controlled trial. *Lancet* 2012; 380: 660–667.
84. Altenburg J, de Graaff C, Stienstra Y, Sloos J, van Haren E, Koppers R, van der Werf T, Boersma W. Effect of Azithromycin Maintenance Treatment on Infectious Exacerbations Among Patients With Non–Cystic Fibrosis Bronchiectasis The BAT Randomized Controlled Trial. *JAMA* 2013; 309: 1251–1259.