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High Prevalence of Respiratory Ciliary Dysfunction in Congenital Heart Disease Patients With Heterotaxy

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Abstract

Background—Patients with congenital heart disease (CHD) and heterotaxy show high postsurgical morbidity/mortality, with some developing respiratory complications. Although this finding is often attributed to the CHD, airway clearance and left-right patterning both require motile cilia function. Thus, airway ciliary dysfunction (CD) similar to that of primary ciliary dyskinesia (PCD) may contribute to increased respiratory complications in heterotaxy patients.

Methods and Results—We assessed 43 CHD patients with heterotaxy for airway CD. Videomicrocopy was used to examine ciliary motion in nasal tissue, and nasal nitric oxide (nNO) was measured; nNO level is typically low with PCD. Eighteen patients exhibited CD characterized by abnormal ciliary motion and nNO levels below or near the PCD cutoff values. Patients with CD aged >6 years show increased respiratory symptoms similar to those seen in PCD. Sequencing of all 14 known PCD genes in 13 heterotaxy patients with CD, 12 without CD, 10 PCD disease controls, and 13 healthy controls yielded 0.769, 0.417, 1.0, and 0.077 novel variants per patient, respectively. One heterotaxy patient with CD had the PCD causing *DNAI1* founder mutation. Another with hyperkinetic ciliary beat had 2 mutations in *DNAH11*, the only PCD gene known to

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cause hyperkinetic beat. Among PCD patients, 2 had known PCD causing *CCDC39* and *CCDC40* mutations.

Conclusions—Our studies show that CHD patients with heterotaxy have substantial risk for CD and increased respiratory disease. Heterotaxy patients with CD were enriched for mutations in PCD genes. Future studies are needed to assess the potential benefit of prescreening and prophylactically treating heterotaxy patients for CD.

Keywords

genomic studies; heart defects; congenital; heterotaxy; nitric oxide; primary ciliary dyskinesia

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Heterotaxy is characterized by randomized variation in the patterning of left-right asymmetry of visceral organs in the thoracic and abdominal cavities. Because left-right asymmetries in the cardiovascular system are essential for efficient oxygenation of blood, heterotaxy patients often need complex cardiac surgeries to repair their structural heart defects. Such cardiac surgeries in heterotaxy patients are associated with disproportionately higher postoperative morbidity and mortality,[1] often with prolonged clinical courses associated with respiratory and multiple serious complications.[2] A retrospective review of 96 patients with congenital heart disease (CHD) operated on at Children's Hospital in Boston showed that 11 of 16 patients dying during the 1-month follow-up period were heterotaxy patients.[3] Another study analyzing surgical outcomes showed 4.8% postoperative mortality for CHD patients with heterotaxy versus 2.4% for CHD patients without heterotaxy.[4] Our retrospective study of CHD patients undergoing cardiac surgery at Children's National Medical Center showed that 84 heterotaxy patients had greater postsurgical mortality, increased postsurgical respiratory complications, and more complicated postsurgical course in comparison with 634 CHD patients without heterotaxy undergoing surgeries with comparable Risk Adjustment in Congenital Heart Surgery-1 scores.[5] These findings suggest that heterotaxy patients have unexplained worse outcomes, with our study indicating an association with increased respiratory complications.

Respiratory complications in patients with CHD are generally attributed to the heart disease, but because airway clearance and left-right patterning both require motile cilia function, it is possible that defects in motile cilia function in the respiratory epithelia could be a contributing factor. The cilium is a complex highly conserved microtubule-based organelle comprising >500 proteins.[6] Cilia are found from single-cell organisms such as *Chlamydomonas* to humans and can be nonmotile or motile.[7] Motile cilia play an important role in mucociliary clearance via coordinated waves of ciliary motion that sweep mucus out of the airway, allowing the removal of cellular debris, microbial contaminants, and other foreign matter. Patients with primary ciliary dyskinesia (PCD) have ineffective mucociliary clearance because of immotile cilia or abnormal ciliary motion.[8] Consequently, PCD patients often exhibit newborn respiratory distress and develop sinopulmonary disease, including bronchiectasis. Because motile cilia in the embryonic node also play a critical role in generating nodal flow required for establishing the left-right body axis and patterning of visceral organ situs,[9] approximately half of PCD patients show complete mirror reversal of visceral organ situs (situs inversus totalis).[10]

Studies in mice showed that mutation in *Dnahc5*, a gene known to cause PCD, can result in a high prevalence of complex CHD and heterotaxy.[11] This mutant is a bona fide model of PCD, exhibiting immotile or dyskinetic respiratory cilia and missing outer dynein arm ultrastructural defect.[12] Whereas 60% of *Dnahc5* mutants are postnatal viable with situs solitus or situs inversus totalis with no heart disease, 40% are prenatal/neonatal lethal with heterotaxy and complex CHD. A retrospective clinical study confirmed a link between PCD and heterotaxy with the finding of 6% incidence of heterotaxy among >300 PCD patients

examined, with 1 of 3 of the heterotaxy patients also exhibiting CHD.[13] Because PCD and heterotaxy are both rare disorders with a prevalence estimated at 1 in 15 000 to 20 000, these findings suggest a mechanistic link between PCD and heterotaxy/CHD.

To investigate whether CHD patients with heterotaxy may have ciliary dysfunction (CD) similar to that of PCD, in this study we assessed airway ciliary function in CHD patients with heterotaxy and used next-generation sequencing to scan for mutations in all 14 genes known to cause PCD. Our studies indicate that heterotaxy patients have significant risk for CD, and this may involve PCD and other cilia genes not known to cause PCD.

Methods

Patient Recruitment

Participants with CHD and heterotaxy were recruited from Children's National Medical Center, National Institutes of Health, University of Pittsburgh, and University of North Carolina with the use of study protocols approved by the respective institutional review boards. Informed consent was obtained from adult subjects or parent of children, with assent also obtained for children >7 years of age. Medical and family history was obtained by use of information from a questionnaire and medical chart review. Blood and/or saliva samples were obtained for genetic analysis. Cardiothoracoabdominal organ situs was assessed by using data obtained by echocardiography, catheterization, and chest and abdominal radiographs and sonographs (see online-only Data Supplement Tables I and II). Cardiac situs was delineated by use of Van Praagh segmental classification.[14]

Nasal Nitric Oxide Measurements

Nasal NO (nNO) measurements were made with use of a chemiluminescence nitric oxide analyzer (CLD88 SP, ECOPHYSICS AG) with established protocols[15] (see online-only Data Supplement Methods and online-only Data Supplement Figure II). For participants >6 years of age, measurements were made by use of the velum-closure technique according to American Thoracic Society/European Respiratory Society guidelines. In patients <6 years of age, NO measurements were made with tidal breath sampling.[16] Some patients with CD and low nNO were reevaluated with a sweat chloride test to exclude cystic fibrosis (online-only Data Supplement Table I).

Nasal Tissue Sampling and Ciliary Motion Analysis

Nasal tissue was collected with use of Rhino-Probe (Arlington Scientific, Springville, UT) curettage of the inferior nasal turbinate. Exclusion criteria included severe bleeding diathesis or conditions such as hemophilia or hereditary hemorrhagic telangiectasia syndrome. The nasal tissue was suspended in L-15 medium (Invitrogen, CA) for videomicroscopy using a Leica inverted microscope (DMIRE2) with a 100× oil objective under differential interference contrast optics. Movies were recorded at 200 frames/s at room temperature by use of a Phantom v4.2 camera (Vision Research, NJ), and digital recordings were evaluated by a blinded panel of coinvestigators (M.L., M.K., R.F., C.W.L., H.O.).

Targeted Sequence Capture and Next-Generation Sequencing

A custom Roche NimbleGen array was designed for targeted capture of coding exons for the 14 PCD genes. Sequence capture and library preparation for SOLiD4 sequencing were performed by use of standard protocols (http://www.nimblegen.com/products/lit/SeqCap_UsersGuide_Service_v3p0.pdf). The Agilent SureSelect human all-exon array were used for sequence analysis of the healthy controls and 9 of the 10 PCD patients. Sequencing data were processed by use of AB Bioscope, with further analysis using CLC Genomics Workbench v4.0.3. Single-nucleotide variants and small insertions/deletions (1–10 bp) were

identified by single-nucleotide polymorphism/deletion-insertion polymorphism detection program at bases with quality scores >20 (99% accuracy), coverage >5 times, and variant allele frequency >20%. Novel coding variants (NCVs) were validated by Sanger sequencing.

Statistical Analysis

The Welch *t* test for unequal variances was used to compare nNO measurements after log transformation of the data. To control for type I error, we applied Bonferroni correction for multiple comparisons (CD versus no-CD heterotaxy patients, healthy controls, or PCD patients; CD-A versus CD-B; no-CD versus healthy control) in each age group, and a probability value of <0.01 was considered statistically significant. If nNO measurements in the CD group were statistically significantly different from no-CD, healthy control, or PCD groups, then we also compared the CD-A and CD-B subgroups with these groups. The χ^2 test was used to test for differences in categorical variables. The analysis of the respiratory symptoms used the Wilcoxon rank-sum test to compare the number of symptoms and the Fisher exact test to compare the frequency of individual symptoms between the CD and no-CD patients. For the DNA-sequencing data, we examined the differences in the presence of NCVs and in the number of NCVs among the 4 groups (heterotaxy patients with CD, heterotaxy patients without CD, PCD patients, and control subjects) using the Fisher exact test and the Kruskal-Wallis test, respectively, and a probability value of <0.025 was considered statistically significant for each test based on the Bonferroni correction. Pairwise comparisons were performed with use of the Fisher exact test or the Wilcoxon rank-sum test only if the comparison in NCVs among the 4 groups showed statistically significant difference. All tests were 2-sided. Analyses were performed with SAS 9.2 (SAS Institute, Cary, NC).

Results

Forty-eight heterotaxy patients were recruited into our study, with 43 having completed the entire study protocol. Among the 43 patients, 20 (47%) had cardiovascular situs defects only (cohort I) and 23 (52%) had cardiovascular and abdominal/lung laterality defects (cohort II) (Figure 1;Table 1). We defined heterotaxy as abnormal or discordant situs involving the cardiovascular system with or without lung or abdominal situs abnormalities (see online-only Data Supplement Methods). This broad definition is based on the known requirement for motile cilia function in left-right patterning of visceral organs in the body. Many of the structural heart defects typically associated with heterotaxy were observed in our patients (Figure 1; online-only Data Supplement Table I).[11,17] Thoracic laterality defects and other disorders were also recorded (see online-only Data Supplement Table II). Patients were further grouped as perioperative for those hospitalized for surgical procedures, or nonperioperative for patients that are either being seen in the Adult Congenital Heart Disease Clinic or undergoing in hospital diagnostic evaluation (see online-only Data Supplement Methods) (Table 1). In addition, we also recruited 18 PCD patients without CHD as disease controls and 25 healthy subjects as healthy controls (Table 1).

Assessment of Ciliary Motion by Videomicroscopy

Nasal tissue was obtained from each patient and assessed for ciliary motion defects by videomicroscopy, and subsequently ciliary beat frequency (CBF) was obtained from analysis of the video sequences (Table 1). Analysis of the CBFs showed no difference in the heterotaxy patients versus healthy controls (P>0.5) (online-only Data Supplement Figure I). However, 2 heterotaxy patients had exceptionally high CBF of 16 Hz (patient 9003) and 19.7 Hz (patient 9002) (Table 1). To assess for other abnormalities in ciliary motion, we examined the pattern of ciliary beat by using slow-motion play back of the video sequence to generate tracings of the ciliary beat (Figure 2). In normal nasal epithelia, the ciliary beat

pattern is characterized by metachronal waves composed of forward and reverse strokes sweeping in a planar motion synchronously across the respiratory epithelium (Figure 2D; online-only Data Supplement Movie I).[18] In PCD patients, a wide range of ciliary motion defects was observed, including immotility, stiff/dyskinetic ciliary motion, and other motion defects (online-only Data Supplement Movie II for PCD patient 9028; online-only Data Supplement Table VI).

Many of the heterotaxy patients also exhibited aberrant ciliary motion (Table 1; online-only Data Supplement Movies III through VI). This included immotile cilia, cilia with stiff/dyskinetic beat (online-only Data Supplement Movie III), incomplete stroke (ciliary beat with decreased amplitude) (online-only Data Supplement Movie IV and Figure 2E), wavy stroke (online-only Data Supplement Movie V; Figure 2F), or asynchronous beat (online-only Data Supplement Movie VI). These ciliary motion defects were found in varying combinations in 18 of the 43 heterotaxy patients (Table 1). In contrast, none of the healthy controls exhibited ciliary motion abnormalities. In heterotaxy patients 9037 and 9002, cilia with abnormal motion were observed in a landscape of mostly immotile cilia, similar to some PCD patients. In heterotaxy patients 9027 and 9046, there was a paucity of cilia (Figure 2B). For patient 9027, the nasal biopsy was performed twice, and in both instances, we found few cilia and ciliary motion was stiff/dyskinetic. In patient 9004, we could find no cilia, either perioperatively at 5.5 months of age or when resampled nonperioperatively at 1.4 years of age (Figure 2C).

After videomicrosocpy, the nasal tissue samples from heterotaxy and PCD patients were processed for electron microscopy to examine cilia ultrastructure. Cross-sectional views suitable for cilia ultrastructure analysis were obtained for 29 of 41 heterotaxy patients, including 11 with CD (Table 1), but no defects were found. In comparison, electron microscopy analysis of 9 PCD patients showed 7 with a variety of ultrastructural defects typically seen with PCD (online-only Data Supplement Table VI).

Assessment of Nasal Nitric Oxide

We measured nasal nNO, which is typically low in PCD patients. Because nNO increased with age, we grouped the nNO measurements into 3 groups: <1 year of age, 1 to 6 years of age, and >6 years of age. Newborns exhibit nNO just a few nanoliters per minute at birth. At 1 to 6 years of age, nNO levels rise to >100 nL/min.[16] In healthy adults, nNO values are usually >200 nL/min.[19] PCD patients 1 to 6 years of age have nNO values <50 nL/min, and those >6 years of age have nNO values <100 nL/min.[20] For patients <1 year of age, nNO cutoff values have not been established, given that nNO values are usually low but dynamically increasing.

For controls, we sampled 25 healthy adults, who exhibited mean nNO of 246±52.2 nL/min (Table 2), and also 18 adult PCD patients, with mean nNO of 16.5±10.5 nL/min (Table 2). These measurements are consistent with values previously reported.[15,19] Assessment of nNO levels in the 43 heterotaxy patients revealed all 18 patients with ciliary motion defects have nNO levels either below or near the PCD cutoff values (Tables 1 and 2; Figure 3). We noted no difference in NO levels in perioperative versus nonperioperative patients (online-only Data Supplement Table III). Significantly, heterotaxy patients with CD had nNO measurements lower than that of healthy controls or heterotaxy patients without CD (no-CD).

Patients >6 Years of Age (Median 18.5 Years)—Nine patients >6 years of age with ciliary motion defects had mean nNO of 95.6±44 nL/min, significantly lower than that of either healthy controls (Table 2; $P<0.001$) or heterotaxy patients without ciliary motion defects (no-CD in Table 2; $P<0.001$). Among the 9 patients with CD, 5 patients had nNO of

64.2±28.7 nL/min, below the 100- nL/min PCD cutoff value, and are referred to as CD type A or CD-A (Table 2). Four had nNO of 134.8±19.6 nL/min, just above the PCD cutoff value and are referred to as CD type B or CD-B (Table 2). The CD-A and CD-B nNO levels were significantly different from the healthy controls ($P<0.001$ and $P=0.002$, respectively), borderline significant from each other ($P=0.02$), and significantly different from the PCD patients ($P<=0.001$) (Table 2).

Patients 1 to 6 Years (Median 2.5 Year)—Four patients exhibiting ciliary motility defects in this age group had combined mean nNO of 52.3±12.4 nL/min. One had nNO of 34 nL/min, below the 50-nL/min PCD cutoff value and thus was categorized as CD-A (Table 2). The other 3 patients with ciliary motion defects exhibited 58.3±3.2 nL/min, just above the 50-nL/min PCD cutoff and thus were categorized as CD-B (Figure 2). Given the small sample size, no significant difference was observed in the mean nNO value in CD (52±12.4 nL/min) versus no-CD (79.8±34.3) ($P=0.12$), but the CD patient nNO measurements were significantly different from those of healthy controls (118.5±59.3; $P=0.008$) and PCD patients (19.7±13.8; $P<0.001$) (Table 2). The single CD-A patient, 9004, was assessed twice, nonperioperatively at 1.4 years of age and perioperatively at 5.5 months of age (see below). The assessment at 1.4 years of age yielded 34 nL/min nNO, within the PCD range (Table 1).

Patients <1 Year of Age (Median 8 Months)—Five heterotaxy patients in this age group with ciliary motion defects had mean nNO of 6.1±2.7 nL/min. This is significantly lower than the 19.6±8.5 nL/min observed in 10 heterotaxy patients with normal ciliary motion (no-CD; Table 2) ($P=0.005$) and indistinguishable from the 7.3±5.7 nL/min observed in PCD patients ($P=0.94$; Table 2). These patients are classified as having CD without further stratification given the lack of PCD cutoff values for this age group. Patient 9004 with 16.5 nL/min nNO was designated as CD-A based on retesting at 1.4 years of age.

Analysis of Laterality Defects, Operative Status, and Sex

The prevalence of CD was greater in the cohort with both cardiovascular and abdominal/lung laterality defects (57%; cohort II) versus those with cardiovascular laterality defects only (25%; cohort I) ($P<0.05$) (Figure 1), but it was not different between the perioperative versus nonperioperative patients (online-only Data Supplement Table IV). In the group >6 years age, CD (type A and B) was observed in 8 nonperioperative versus 1 perioperative patient (Table 1). Fifty percent of the CD patients were male, in comparison with only 28% males among patients with no CD, but this difference was not significant given the small sample size ($P=0.14$; online-only Data Supplement Table IV).

Analysis of Respiratory Symptoms

Analysis of the prevalence of respiratory symptoms showed 24 of the 43 (56%) patients have history of at least 1 chronic respiratory manifestation. This was not significantly different between the CD versus non-CD cohort with 10 (56%) of the 18 CD versus 14 (56%) of the 25 no-CD patients reporting at least 1 respiratory symptom ($P>0.1$) (Table 3; online-only Data Supplement Table V). Comparison of the number of perioperative versus nonperioperative patients with respiratory symptoms also showed no difference (online-only Data Supplement Table III). Among the 9 patients >6 years of age with at least 1 respiratory manifestation, 5 patients with CD had more symptoms in comparison with the 4 no-CD patients (medians are 4 and 2, respectively; $P=0.024$). Sequelae associated with PCD (neonatal respiratory distress, bronchiectasis, or recurrent otitis media) were present in 5 of the 8 CD patients >6 years of age, but none of the 7 no-CD patients ($P=0.026$).

Sequencing Analysis of 14 Known PCD Genes

We used next-generation sequencing with targeted exon capture to interrogate for mutations in all 325 coding exons and flanking intron sequences of the 14 genes known to cause PCD (see online-only Data Supplement Methods).[21–23] Sequencing analysis was performed for 13 heterotaxy patients with CD, 12 heterotaxy patients without CD, 10 PCD disease controls, and 13 healthy controls (Table 4). An average sequencing coverage of 42 times was obtained encompassing 99% to 100% of the bases in 12 of the 14 PCD genes (online-only Data Supplement Table VII). Over 1000 (n=1012) coding variants were identified in the 48 individuals, including nonsynonymous single-nucleotide variants, insertions/deletions, and variants altering splice junctions. Filtering to remove variants in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) and 1000Genomes (http://www.1000genomes.org) databases yielded 42 novel single-nucleotide variants, 34 of which validated by Sanger sequencing (Table 4, online-only Data Supplement Table VIII).

Four PCD patients were found to have 2 known PCD-causing mutations (online-only Data Supplement Table VIII). Patient 9028 and 9098 were homozygous for a *CCDC39* mutation, 1972delA, recently identified to cause PCD.[22] Patient 9028 showed ultrastructural defects consistent with *CCDC39* mutations (online-only Data Supplement Table VI). PCD patients 564 and 1401 were both heterozygous for a known PCD causing *CCDC40* mutation (248delC).[23] In patient 1401, a second novel *CCDC40* mutation was observed, which was confirmed to be in *trans*. Cilia ultrastructural abnormalities expected for CCDC40 mutations were observed (online-only Data Supplement Table VI). In patient 1174, 2 novel *DNAH5* mutations were also found in *trans*, and, consistent with a role in disease, electron microscopy analysis showed outer dynein arm defects (online-only Data Supplement Table VI). Sequencing analysis of the 13 heterotaxy patients with CD revealed 1 individual (9002) heterozygous for the *DNAI1* founder mutation, IVS1+2_3insT, known to cause PCD.[24] Another patient (9026) had 3 novel mutations, 2 in *DNAH5* and1in *DNHA11*. Patient 9003 exhibited 2 *DNAH11* mutations. This was associated with hyperkinetic ciliary beat, a phenotype unique to PCD arising from *DNAH11* mutations.[23]

Overall, the highest incidence of NCVs was observed in the PCD patients, 1 NCV/subject (Figure 4; online-only Data Supplement Table IX), followed by heterotaxy patients with CD yielding 0.769 NCV/subject, then heterotaxy patients with no-CD with 0.417 NCV/subject (Figure 4; online-only Data Supplement Table IX). In contrast, the healthy controls had a single variant, yielding 0.077 NCV/subject. Statistical analysis of the percentage of patients with NCV and the mean number of NCV per subject showed significant differences for heterotaxy patients with CD and the PCD patients in comparison with controls (Figure 4; online-only Data Supplement Table IX). This is not accountable by differences in racial composition, because Fisher exact test for race-ethnicity between the control subjects versus heterotaxy patients with CD yielded P=0.33 (Table 4; online-only Data Supplement Table VIII).

Discussion

We showed that 18 of 43 (42%) of patients with CHD associated with heterotaxy have CD. The abnormal ciliary motion is similar to that seen with PCD, including dyskinetic, stiff, or wavy ciliary beat. The latter may be comparable to the whiplike motion reported for some PCD patients.[18] Two patients have hyperkinetic ciliary beat reminiscent of ciliary motion defects caused by *DNAH11* mutations.[25] Importantly, all heterotaxy patients with CD also had nNO levels either below (CD-A) or bordering (CD-B) the PCD cutoff values. Postsurgical trauma cannot account for the finding of CD, because CD was not correlated with operative status. The risk for CD was higher in patients with laterality defects involving both cardiovascular and lung/abdominal organs. These findings suggest CHD patients with

heterotaxy may have CD overlapping with that of PCD. Because PCD and heterotaxy each have a prevalence of 1 in 15 000 to 20 000 live births, this is not likely by chance, but it may reflect the common requirement for motile cilia in mucociliary clearance and left-right patterning.

Ciliary Dysfunction in Heterotaxy versus PCD Patients

Although we observed that heterotaxy patients with CD have low nNO, their mean nNO values were higher than those of PCD patients, even among CD-A patients. Heterotaxy patients with CD also have higher CBFs than PCD patients do. Moreover, none of the 11 heterotaxy patients with CD examined by electron microscopy exhibited cilia ultrastructural defects. In contrast, a recent study of a large cohort of PCD patients showed 50% have cilia ultrastructural defects.[26] Together, these findings suggest heterotaxy patients with CD may have a variant form of CD differing from PCD. Nevertheless, studies in mouse models have shown that mutations in PCD genes (*Dnahc5*, *Dnaic1, Dnahc11*) can cause CHD with heterotaxy.[11,27,28] However, these may be underrepresented among heterotaxy patients, because all *Dnahc5* and *Dnaic1* mutants with heterotaxy die prenatally or neonatally from complex CHD. Similarly, *Dnahc11 iv/iv* mutants exhibit a 40% incidence of CHD prenatally, but only 5% among term fetus or viable adult animals.[28,29]

Mutations in PCD Genes

Sequencing analysis of 48 subjects showed heterotaxy, and PCD patients were significantly enriched for NCVs in PCD genes in comparison with healthy controls. We noted that 4 of the 10 PCD patients did not have mutations in any of the 14 PCD genes, suggesting additional novel PCD genes are yet to be identified. One heterotaxy patient with CD had a *DNAI1* founder mutation known to cause PCD, whereas 4 PCD patients (3 unrelated) had 2 known PCD causing *CCDC39 or CCDC40* mutations. Because PCD is a recessive disorder, PCD-causing mutations are expected to be homozygous or compound heterozygous. Consistent with this, 3 PCD patients were homozygous or compound heterozygous for PCD causing *CCDC39 or CCDC40* mutations. In contrast, no heterotaxy patients had 2 known PCD mutations.

For 5 heterotaxy patients with CD and 2 PCD patients, only a single heterozygous PCD gene mutation was observed. This included PCD patient 564 with a known disease-causing *CCDC40* mutation, and heterotaxy patient 9002 with the *DNAI1* founder mutation. In heterotaxy patient 9026, a *DNAH5*/*DNAH11* double-heterozygous mutation was observed. We hypothesize that PCD or CD in heterotaxy patients may arise from 2 mutations, 1 in each of 2 different cilia-related genes. This may involve the combined effects of mutations in PCD genes and other cilia-related genes not associated with PCD. Such multigenic etiology and genetic heterogeneity may underlie the phenotypic differences in the ciliary dysfunction seen in heterotaxy versus PCD patients. This genetic model is supported by studies in *Chlamydomonas* using stable diploids that has shown noncomplementation of unlinked genes required for ciliogenesis.[30,31]

Heterotaxy patient 9003 had 2 *DNAH11* mutations, both predicted to be damaging. Although we could not verify if these mutations were in *cis* given their >143 kb genomic distance (and parental DNA were unavailable), the finding of hyperkinetic ciliary beat and normal cilia ultrastructure is consistent with *DNAH11* mutations. This patient also exhibited sequelae consistent with PCD (newborn respiratory distress, otitis media, recurrent pneumonia, and bronchiectasis). Although this patient had normal cardiac situs, this was associated with abnormal ipsilateral positioning of the aorta, left-sided IVC, and polysplenia. This compares favorably with the finding that 95% of viable *Dnah11 iv/iv* adult

and newborn mice have atrial situs solitus or atrial situs inversus, but mostly without cardiac lesions and with 1 of 3 exhibiting abnormal spleens.[29]

Respiratory Diseases in Heterotaxy Patients With Ciliary Dysfunction

Respiratory symptoms and disease were increased in heterotaxy patients with CD >6 years of age. Neonatal respiratory distress and bronchiectasis, respiratory complications commonly associated with PCD, were found only in heterotaxy patients with CD. Although we did not find a significant increase in respiratory symptoms and disease in heterotaxy patients with CD from the younger age groups, most of these patients were hospitalized for cardiac surgery, and their respiratory symptoms are likely largely attributed to their heart disease. Moreover, respiratory illnesses, such as bronchiectasis, would not manifest until later in life.

We note CD in the respiratory epithelia also has been reported in patients with Leber congenital amaurosis (LCA),[32] a ciliopathy involving cone-rod dystrophy due to defects in the connecting cilium required for photoreceptor biogenesis. Patients exhibit “rarefaction of ciliated cells,” which also was observed in many of our heterotaxy patients with CD. Analysis of the LCA patient nasal tissue showed abnormal ciliary beat pattern, and 6 of 7 LCA patients had a clinical history of recurrent inflammatory diseases of the airways. These findings suggest CD and increased respiratory disease may have relevance for other cilia-related disorders.

Future Prospects

Overall, our studies suggest CHD patients with heterotaxy have substantial risk for CD and respiratory disease. This may involve mutations in novel and known PCD genes. These findings suggest CHD patients with heterotaxy may benefit from preoperative screening for CD. Further studies are needed to evaluate whether therapies enhancing mucus clearance may reduce respiratory complications and improve postsurgical outcome for CHD patients with CD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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CLINICAL PERSPECTIVE

Heterotaxy patients have some of the most complex congenital heart disease. Although surgical advances now allow palliation and repair of even the most complex structural heart defects, the clinical management of heterotaxy patients remains challenging. Heterotaxy patients have unexplained higher morbidity and mortality, often with long clinical courses and increased respiratory complications. Although this higher morbidity and mortality is usually attributed to the heart disease, the underlying cause remains largely unknown. Because motile cilia are required both for left-right patterning and for mucociliary clearance in the airway, we hypothesize that heterotaxy patients may have risk for airway clearance defects that overlap with that of primary ciliary dyskinesia (PCD). PCD is a heritable disorder characterized by both laterality defects and also sinopulmonary disease arising from airway cilia motility defects that compromise mucus clearance. Using 2 simple tests normally used for PCD assessment, we showed that 42% of heterotaxy patients with CHD have airway ciliary dysfunction (CD). This included the observation of abnormal cilia motility by videomicroscopy of nasal scrapes, and also low nasal nitric oxide (NO) levels similar to the low NO levels seen with PCD. Furthermore, sequencing analysis showed that heterotaxy patients with CD are significantly enriched for novel coding variants in genes known to cause PCD. Together these findings suggest that CHD patients with heterotaxy may benefit from preoperative screening for CD, with pulmonary therapy to be provided prophylactically to enhance airway clearance in patients with CD. Such a change in the standard of care may improve outcome and reduce morbidity/mortality in CHD patients with heterotaxy.

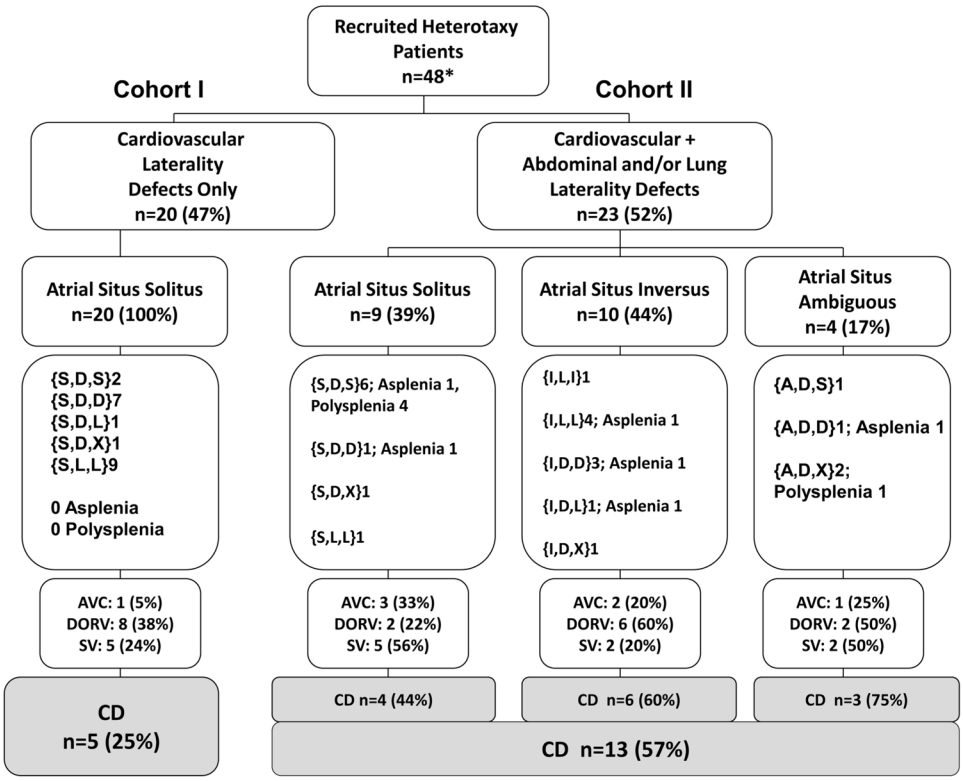

Figure 1.

Laterality and cardiovascular defects in heterotaxy patients. Patients recruited for study; 5 did not complete study. AVC indicates atrioventricular canal; DORV, double outlet right ventricle; SV, single ventricle; CD, ciliary dysfunction.

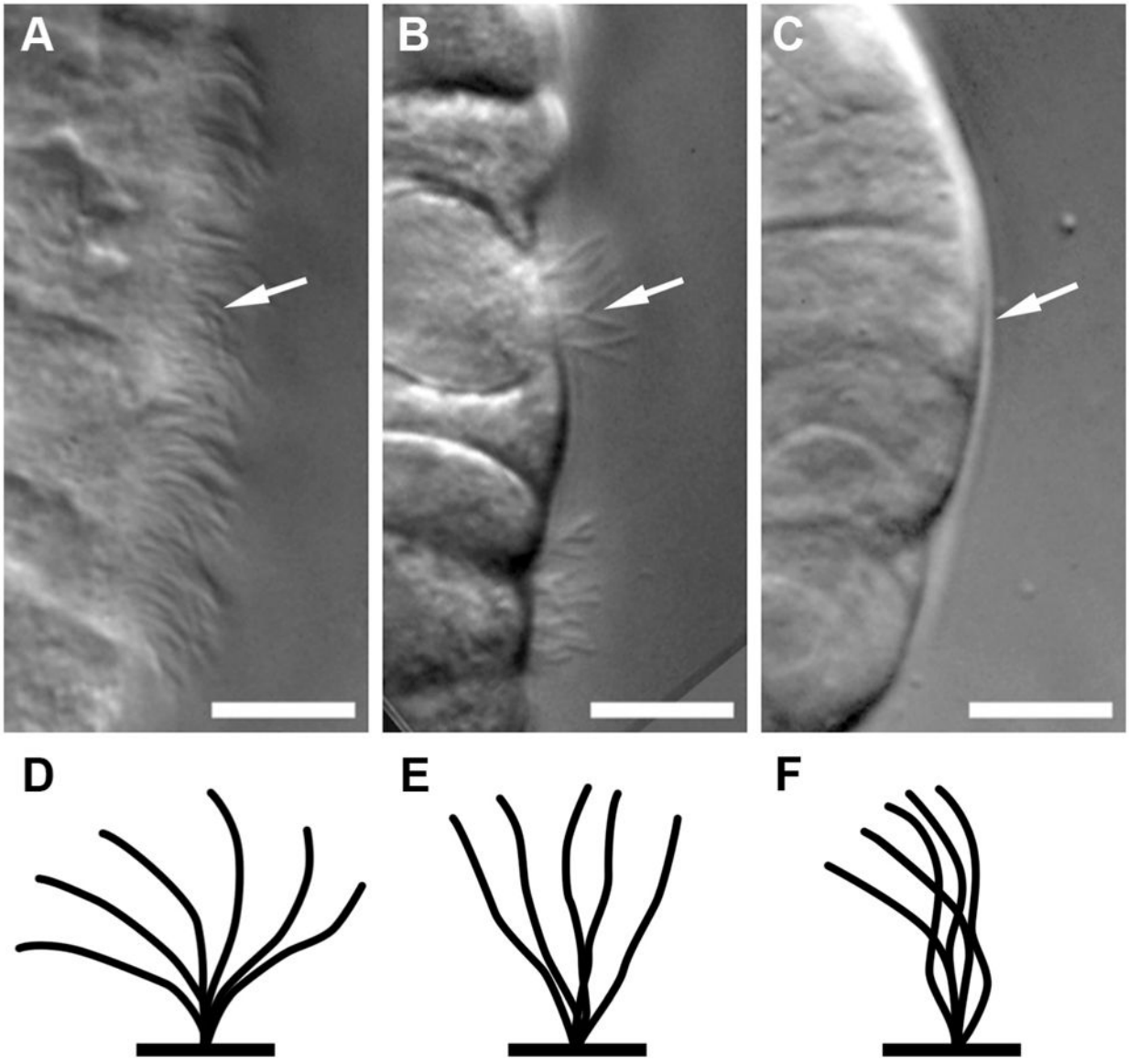

Figure 2.

Ciliary motion in nasal epithelia from heterotaxy and PCD patients. **A**, Abundant cilia (arrow) were observed in patient 9033 exhibiting normal ciliary motion. **B**, Paucity of cilia (arrow) in 9027 with CD. **C**, No cilia found in nasal epithelia (arrow) of 9004. **D**, Healthy control showing normal motion with full forward and recovery strokes (online-only Data Supplement Movie I). **E**, PCD patient 9028 has stiff motion with shortened stroke and minimal ciliary bending. **F**, Patient 9011 with heterotaxy and CD exhibit shortened forward stroke and wavy recovery stroke with limited bending of the distal ciliary exoneme (online-only Data Supplement Movie V). Scale bar=10 μm.

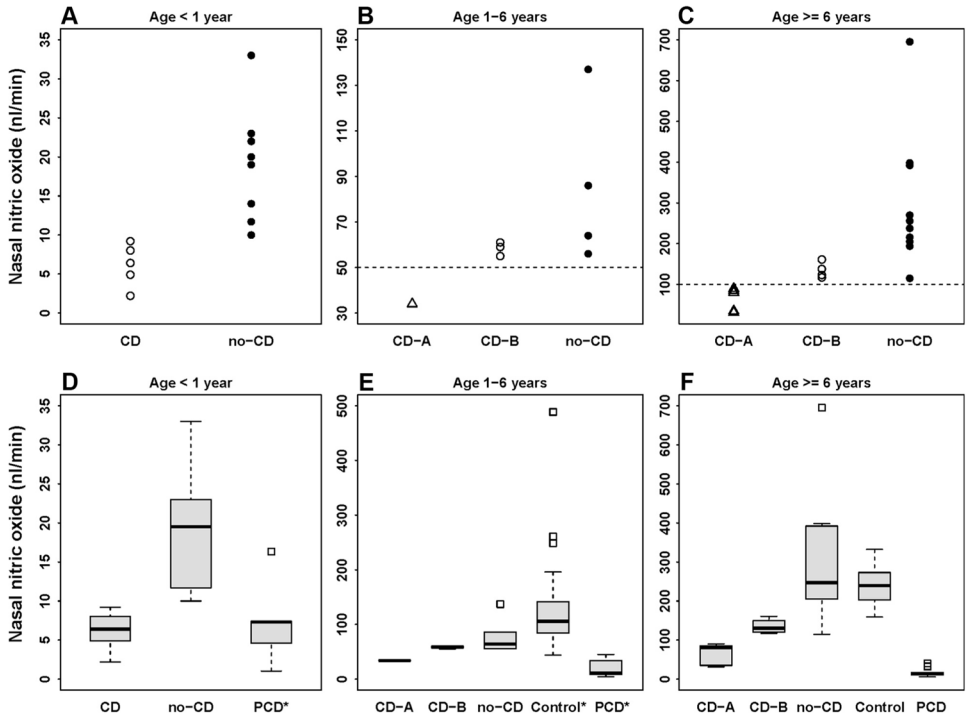

Figure 3.

Nasal nitric oxide measurements in heterotaxy patients. **A** through **C**, nNO measurements from each patient are plotted in 3 age groups. **D** through **F**, Box plots show interquartile range (IQR) from 25 to 75th percentile, with median denoted by bold line. Whiskers denote minimum-maximum value not >1.5 times the IQR value, with outlying values indicated by squares. *The nNO values for PCD patients <1 and 1 to 6 years of age were adapted from Chawla et al.[16] CD indicates ciliary dysfunction; PCD, primary ciliary dyskinesia.

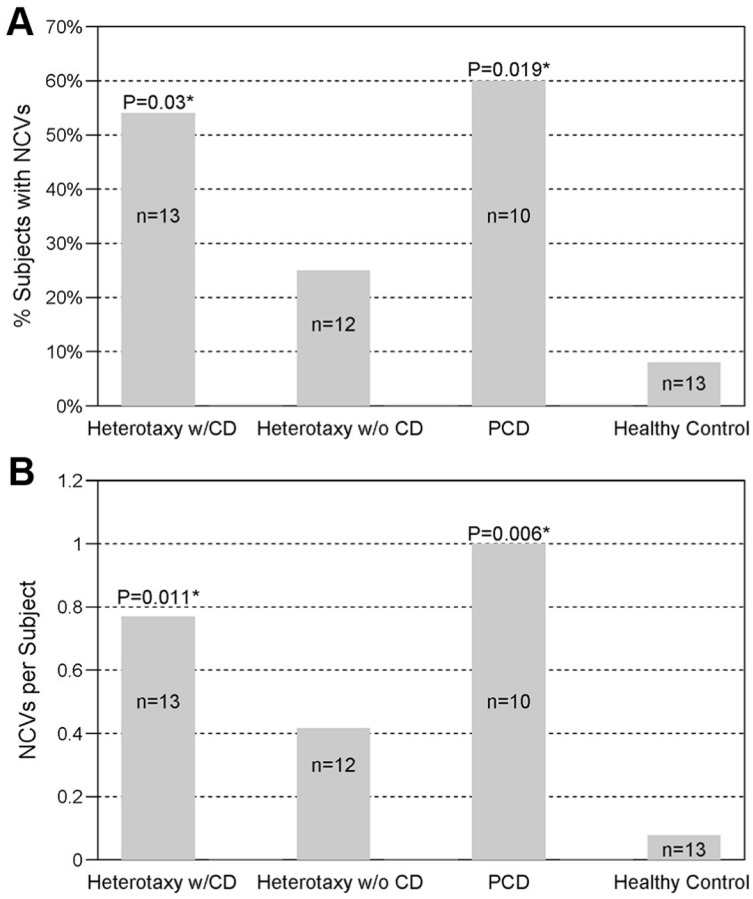

Figure 4.

Novel coding variants in PCD genes. More NCVs in PCD genes were observed in heterotaxy patients with CD and PCD patients than healthy controls. *Probability values obtained in comparison with controls. Statistical analysis was performed with the Fisher exact test (**A**) or the Kruskal-Wallis test followed by Wilcoxon rank sum test (**B**) with $P<0.025$ considered significant based on Bonferroni correction.

Table 1

Cilia Analysis and NO Measurements

Patients		Age	nNO, nL/min	Cilia Analysis		Cohort[†]	Cilia Function	EM[‡]
				Motion[*]	CBF(Hz)			
>6 y of age								
Perioperative								
9011	Asplenia	12 y	85	e	4.9	II	CD-A	Y
9013		7 y	115	a	4.7	I	no-CD	Y
9016		19 y	216	a	3.9	I	no-CD	Y
9046		9 y	238	a, j	n/a	II	no-CD	N
Nonperioperative								
9003	Polysplenia	26 y	31	b, d, e	16	II	CD-A	Y
9020	?	46 y	90	b, d	7.7	I	CD-A	Y
9032	Normal	19 y	80	d	3.6	I	CD-A	Y
9037	Normal?	18 y	35	b, c	9.5	II	CD-A	Y
9008	Normal	10 y	138	b, g	4.2	I	CD-B	N
9026	Polysplenia	18 y	161	d	6.8	II	CD-B	Y
9027	Left Isomerism	6.5 y	123[§]	b, j	6.2	II	CD-B	N
9031	Normal	15 y	117	b	7.2	II	CD-B	N
9009		30 y	205	d	5.1	I	no-CD	Y
9019		44 y	392	a	7.5	I	no-CD	N
9033		29 y	270	d	5.1	I	no-CD	Y
9035		28 y	695	a	4.7	II	no-CD	Y
9036		25 y	194	a	4.9	I	no-CD	Y
9038		50 y	398	a	3.2	I	no-CD	N
9042		12 y	256	a	7.1	I	no-CD	N
1–6 y of age								
Perioperative								
9010	?	2 y	59	d, f	4.9	I	CD-B	N
9017	Normal	5.9 y	55	d, f	8.7	II	CD-B	Y
9014		3 y	64[§]	a	3	I	no-CD	Y

				Cilia Analysis				
Patients		**Age**	**nNO, nL/min**	**Motion***	**CBF(Hz)**	**Cohort†**	**Cilia Function**	**EM‡**
9022		2.5 y	56	a	8.1	I	no-CD	ND
9024		2 y	56	a	8.8	II	no-CD	Y
Nonperioperative								
9004	Asplenia	1.4 y	34§	j	n/a	II	CD-A	N
9006	Unknown	2.5 y	61	d, e	6	II	CD-B	Y
9001		5 y	141	a	7.1	II	no-CD	Y
9007		18 mo	86§	a, g	10.7	II	no-CD	Y
<1 y of age								
Perioperative								
9004	Asplenia	5.5 mo	16.5	j	n/a	II	CD	N
9015	Normal	9 mo	2.2	d, e	6.7	II	CD	Y
9018	Normal	3 mo	8.0	b, e	9.5	I	CD	N
9023	Normal	7 mo	4.9	d	7.5	II	CD	ND
9043	Normal	14 d	9.2	e	n/a	II	CD	Y
9005		24 d	11.7	a	8.7	II	no-CD	N
9025		10 d	10	a	8.5	II	no-CD	N
9039		18 d	19	a	11	I	no-CD	Y
9044		9 mo	22	a	12.2	I	no-CD	Y
9045		1.5 mo	33	a	5.7	I	no-CD	Y
9049		0.8 mo	14	a	9.7	I	no-CD	Y
Nonperioperative								
9002	Asplenia	17 d	6.4	b, c	19.7	II	CD	Y
9021		3 mo	23	a	5	II	no-CD	Y
9029		5 mo	33	a	8.3	II	no-CD	Y
9040		15 d	20	a	7.2	II	no-CD	Y
9041		8 d	10	a	6	I	no-CD	Y

CBF indicates ciliary beat frequency; NO, nitric oxide; nNO, nasal nitric oxide; PCD, primary ciliary dyskinesia; CD, ciliary dysfunction; CD-A, ciliary dysfunction with nNO below PCD cutoff; CD-B, ciliary dysfunction with reduced nNO value bordering PCD cutoff; EM, electron microscopy.

* a indicates normal; b, stiff/dyskinetic; c, immotile; d, incomplete stroke; e, wavy stroke; f, Asynchronous; g, excessive mucus present; j, few/no cilia seen.

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† I indicates cardiovascular situs abnormalities only; II, cardiovascular with thoracic and/or abdominal situs abnormalities.

‡ Cilia EM obtained (yes [Y]) or not adequate/not available (no [N]).

§ nNO remeasured.

Table 2

nNO Measurements in Heterotaxy Patients

	Heterotaxy Patients					
Age	**All With CD (n=18)**	**CD-A (n=6)**	**CD-B (n=12)**	**no-CD (n=25)**	**Healthy Controls**	**PCD**
<1 y (n=15)	6.1±2.7 (n=5)	. . .		19.6±8.5 (n=10)	. . .	7.3±5.7 (n=5)*
	P=0.005†					
	P=0.94‖					
1–6y(n=9)	52.3±12.4 (n=4)	34 (n=1)	58.3±3.2 (n=3)	79.8±34.3 (n=5)	118.5±59.3 (n=90)†	19.7±13.8 (n=17)*
	P=0.12†				*P*=0.097*	
	P=0.008§		*P*<0.001§			
	P<0.001‖		*P*<0.001‖			
≥6y(n=19)	95.6±44.0 (n=9)	64.2±28.7 (n=5)	134.8±19.6 (n=4)	297.9±164.1 (n=10)	246.3±52.2 (n=25)	16.5±10.5 (n=18)
		P=0.02¶		*P*=0.55#		
	P<0.001†	*P*<0.001†	*P*=0.002†			
	P<0.001§	*P*=0.003§	*P*<0.001§			
	P<0.001‖	*P*=0.001‖	*P*<0.001‖			

Values for nNO are in nL/min. nNO indicates nasal nitric oxide; PCD, primary ciliary dyskinesia; CD, ciliary dysfunction; CD-A, ciliary dysfunction with nNO below PCD cutoff; CD-B, ciliary dysfunction with reduced nNO value bordering PCD cutoff; and no-CD, without CD.

*Adapted from Chawla et al.[16]

†Welch *t* test comparison of CD with no-CD.

§Welch *t* test comparison of CD with controls.

‖Welch *t* test comparison of CD with PCD patients.

¶CD-A vs CD-B comparison.

#no-CD vs healthy control comparison.

Table 3

Respiratory Manifestations in Heterotaxy Patients*

Patients >6 y of Age		Age, y	Sex	Recurrent Otitis Media	Recurrent Lower Respiratory Illnesses	Neonatal Respiratory Distress	Chronic Wet Cough	Chronic Nasal Congestion	Chronic Sinusitis	Respiratory Insufficiency/Tracheotomy	Bronchiectasis
Perioperative											
CD-A	9011	12	F	N	N	N	N	N	N	N	N
no-CD	9013	7	F	N	Y	N	N	N	N	N	N
no-CD	9016	19	F	N	N	N	N	N	N	N	N
no-CD	9046	9	F	Y	N	N	N	N	N	N	N
Nonperioperative*											
CD-A	9003	26	F	Y	Y	Y	Y	N	Y	N	Y
CD-A	9020	46	F	Y	Y	N	Y	N	N	N	N
CD-A	9032	19	M	N	N	N	N	N	N	N	N
CD-A	9037	18	M	Y	N	Y	Y	Y	Y	N	N
CD-B	9008	10	M	N	N	N	N	N	N	N	N
CD-B	9026	18	F	N	N	N	N	N	N	N	N
CD-B	9027	6.5	F	N	Y	Y	Y	Y	N	N	N
CD-B	9031	15	M	Y	Y	N	N	Y	Y	N	N
no-CD	9009	30	F	N	N	N	N	N	N	N	N
no-CD	9019	44	M	N	Y	N	Y	N	Y	N	N
no-CD	9033	29	M	N	N	N	N	N	N	N	N
no-CD	9035	28	F	N	N	N	N	N	N	N	N
no-CD	9036	25	M	N	N	N	N	N	Y	N	N
no-CD	9038	50	F	N	Y	N	N	N	Y	N	N
no-CD	9042	12	F	N	N	N	Y	Y	N	N	N

Y indicates with symptoms; N, without symptoms; PCD, primary ciliary dyskinesia; CD, ciliary dysfunction; CD-A, ciliary dysfunction with nNO below PCD cutoff; CD-B, ciliary dysfunction with reduced nNO value bordering PCD cutoff; and no-CD, without CD.

*Wilcoxon rank sum test show P=0.024 for number of respiratory manifestations in nonperioperative CD vs no-CD patients.

Table 4

Novel Sequence Variants and Mutations in PCD Genes

Patient	Ethnicity	Function	Gene	Base Change*	Amino Acid†
9002	White	CD	*DNAI1*	IVS1+2_3insT†	Truncation
9003	Black	CD	*DNAH11*	4520A>C	Q1507P†
			DNAH11	9397G>A	E3133K†
9004	Asian	CD	*TXNDC3*	1630G>A	A544T†
9006	White	CD	*CCDC39*	626C>G	A209G†
9008	Black	CD	None		
9011	White	CD	None		
9015	White	CD	*LRRC50*	1294G>A	E432K
9017	Black	CD	None		
9018	Black	CD	None		
9026	Black	CD	*DNAH11*	9203A>G	E3068G†
			DNAH5	11140A>G	I3714V
			DNAH5	638C>A	P213Q†
9027	White	CD	None		
9031	White	CD	None		
9037	Black	CD	*DNAI1*	1579T>G	S527A†
9005	Black	no-CD	None		
9007	Black	no-CD	None		
9009	White	no-CD	None		
9012	Black	no-CD	None		
9016	White	no-CD	None		
9019	Black	no-CD	*DNAH5*	6710A>G	N2237S
9024	Black	no-CD	None		
9025	Asian	no-CD	*DNAI1*	1795G>A	A599T†
			DNAI1	2054T>C	L685P†
9033	White	no-CD	*CCDC39*	1865A>G	E622G
			DNAI1	1177G>A	V393 mol/L

Patient	Ethnicity	Function	Gene	Base Change*	Amino Acid†
9035	White	no-CD	None		
9040	Black	no-CD	None		
9068	White	no-CD	None		

CD indicates ciliary dysfunction; no-CD, without CD; and PCD, primary ciliary dyskinesia.

*Each allele listed, with homozygous mutation listed twice.

†*DNAI1* founder mutation.[24]